AGING OF METALLIC HYDROXIDE-COATED SAND IN PARTICLE FILTRATION

By

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Aging of metallic hydroxide-coated granular filter media in response to water and wastewater conditions was investigated using aluminum hydroxide-coated sand as the model system. Al-coated sand was exposed to treated wastewater or pH-adjusted tapwater in continuous flow-through columns to assess the effects of biogrowth and influent pH, respectively. Performance of filter media was evaluated in terms of *Escherichia coli* removal. The fluorochrome 4',6-diamidino-2-phenylindole (DAPI), which was found to have no significant effect on bacterial surface properties and interaction with the surface-modified granular media, was used to tag the test bacteria. Exposure to tapwater or wastewater resulted in steady decline of removal capacity of Al-coated sand to that of uncoated sand in 1-4 months. In the case of exposure to wastewater, a "conditioning effect" (increase in removal capacity) similar to that found in slow sand filters was observed after short-term (days to weeks) exposure. Prolonged exposure (weeks to months) to wastewater resulted in progressive biogrowth and corresponding decline of removal capacity. Zeta potential and metal content of the test sands decreased initially (hours to days), then remained relatively constant. No apparent correlation was found between these surface characteristics and removal capacity. In the case of exposure to tapwater, removal capacity of Al-coated sand was strongly influenced by fluid pH. The order of magnitude of the pH effects was pH 5.0 > pH 9.0 > pH 7.0, although the rates of decline after an initial 24-hr period were similar for all 3 pH levels. These pH effects appeared to be consistent with the pH-dependent solubility of aluminum hydroxide. The resultant information on the stability and performance of the surface coating in response to influent conditions is particularly useful for assessing the technological potential and economical implications of surface modification of filter media for water and wastewater treatment.

CHAPTER 1
INTRODUCTION

Natural granular media such as sand and diatomaceous earth have been used for centuries to purify water and, more recently, to treat wastewater. In current practice, chemical flocculants are often added to the water to modify the characteristics of the particulate contaminants to enhance their interactions with the granular filter media. These chemicals include hydrolyzing metal salts (e.g., ferric chloride, aluminum sulfate), natural inorganic and organic polymers (activated silica, starch), and synthetic organic polymers.

Removal of fine particles such as bacteria and viruses is difficult, however, since these biocolloids carry a negative surface charge in the pH range of natural waters (Loder and Liss, 1985; Marshall, 1976) and most filter media (e.g., sand, diatomaceous earth) are also negatively charged in this pH range. An emerging strategy for overcoming this electrostatic interaction barrier is to make the surface of filter media more electropositive, thus facilitating colloidal attachment. This new approach has been shown to be effective in enhancing particle filtration (Bales et al., 1991; Farrah & Preston, 1991; Farrah et al., 1991; Ho et al., 1991; McCaulou et al., 1994; Scholl & Harvey, 1992; Stenkamp & Benjamin, 1994). Coatings of metallic hydroxides on filter media increase removal of bacteria, viruses and turbidity from water and wastewater (Ahammed and Chaudhuri, 1996; Gerba et al., 1988; Lukasik et al., 1996; Mills et al., 1994).

When coated filter media are placed in service, the metallic hydroxide surface will be subject to dissolution, attrition, change in surface area and roughness, and chemical or biological fouling, all of which can influence interaction of the surface with colloids. Thus far, these aging effects have been overlooked. Ahammed and Chaudhuri (1996) reported that coatings of iron hydroxide or iron and aluminum hydroxide on sand continued to effectively remove fecal coliforms from water after 50 days of intermittent operation. The effect of continuous exposure of coated media to water or wastewater was not investigated, however. Overgrowth of microorganisms on the media could diminish the effects of surface modification. The stability of surface coatings could be influenced by influent pH, since the dissolution behavior of metallic hydroxides (e.g., aluminum hydroxide) is pH-dependent (Snoeyink & Jenkins, 1980). These factors (i.e., biogrowth, pH, etc.) must be investigated and understood for surface-modified media to be used in prototype filtration systems.

Assessment of their effects on the performance of surface-modified filter media requires some means of bacterial tagging to distinguish test bacteria from biofilm bacteria. Fluorochromes such as Acridine Orange (Hobbie et al., 1977) and 4’, 6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980) can serve as simple, direct, and safe tagging agents in microbial transport studies of short-term nature (e.g., Bales et al., 1995; Harvey et al., 1989; Harvey & Garabedian, 1991; Harvey et al., 1993; Scholl & Harvey, 1992). The effects of the staining procedure on the surface properties and transport characteristics of microorganisms have not been investigated and understood, however.

The objectives of this work were to: (1) assess the effect of long-term exposure to water and wastewater on the stability, surface characteristics, and performance of metallic hydroxide-coated granular media; (2) correlate the performance of surface-modified filter media with influent characteristics (pH) and biogrowth on the surface; and (3) identify a suitable fluorochrome as bacterial tagging agent that does not significantly affect bacterial surface properties and interaction with granular media (sand). Long-term, laboratory-scale column experiments were carried out using aluminum hydroxide-coated sand as the model system. Al-coated sand was exposed to treated wastewater or pH-adjusted tapwater in continuous flow-through columns. Performance of filter media was measured in terms of *Escherichia coli* removal in subsequent batch and column tests. Biogrwoth, metal content, and zeta potential of the test sands were also measured in parallel to assess their effects on bacterial removal. Biofilms were visualized by scanning electron microscopy (SEM). Effect of staining on bacterial surface properties was characterized by changes in zeta potential and microbial adhesion to hydrocarbons (MATH). Effect of staining on bacterial interaction with granular media (sand) was assessed based on batch removal of pure cultures of *Escherichia coli* and *Acinetobacter* sp. as well as natural wastewater bacteria. The fluorochrome 4',6-diamidino-2-phenylindole (DAPI) was found to have no significant effect on bacterial surface properties and interaction with sand and was thus used to tag the test bacteria.

The removal capacity of Al-coated sand declined steadily in response to continuous exposure to wastewater or tapwater. Zeta potential and metal content of the filter media were also affected by exposure to wastewater, although no apparent correlation was found between these surface characteristics and removal capacity. In the case of exposure to wastewater, a "conditioning effect" (increase in removal capacity) similar to that found in slow sand filters was observed after short-term (days to weeks) exposure. Prolonged exposure (weeks to months) resulted in progressive decline of removal capacity. This decline was accelerated in response to biogrowth on the filter media. In the case of exposure to tapwater, no “conditioning effect” was observed. The removal capacity of Al-coated sand was strongly influenced by influent pH. Based on the experimental results, stability and effective lifetime of the surface coating in response to influent conditions were determined.

This work has gained much-needed understanding of the aging process of metallic hydroxide-coated natural granular media (sand). Understanding of the aging process can prompt strategies to improve coating stability and performance. This understanding is also useful for assessing the technological potential as well as economic implications of surface modification of filter media for water and wastewater treatment.

CHAPTER 2
LITERATURE REVIEW

Introduction

Biofilm formation is a ubiquitous phenomenon (Flemming, 1993) arising from the ability of microorganisms to attach to and grow tenaciously on solid surfaces (Zottola, 1994). The majority of microorganisms on earth are believed to live in biofilms (Costerton et al., 1987). Biofilm formation represents a survival strategy for microorganisms adapting to their habitat environments. Biofilms afford microorganisms greater access to substrates and nutrients (Freeman & Lock, 1995; Rittmann, 1990), protection from the toxic and inhibitory effects of antimicrobial agents (Fletcher, 1991; Van Loosdrecht, et al., 1990), and benefit as a buffer to changing environmental conditions (Geesey et al., 1992). Fixed cultures are less sensitive to changes in environmental conditions than suspended cultures (Flemming, 1993; Stevens, 1988).

The role of bacterial attachment was first recognized in association with the fouling and corrosion of solid surfaces submerged in seawater (Zobell & Allen, 1935). Since then, many beneficial as well as detrimental effects of biofilm formation have been recognized and studied. The growth of biofilms often determines the rate at which environmental contaminants are removed or degraded in natural aquatic environments (Gantzer et al., 1988). Biofilms play an essential role in environmental protection, such as in water and wastewater treatment (Flemming, 1993), in the cleanup of toxic and hazardous wastes (Bouwer, 1985), and more recently in air purification systems

(Flemming, 1993). Biofilm formation on surfaces of ceramic, metallic and plastic materials can lead to biofouling or biocorrosion, causing serious problems in water and wastewater treatment systems (Flemming, 1987; Ridgway et al., 1984; Sand & Bock, 1991), drinking water distribution systems (Emde et al., 1992; LeChevallier et al., 1987), industrial water systems (Geesey, 1991), and food processing systems (Zottola, 1994).

Biofilm formation at the solid-liquid interface during the filtration process alters the physical and physicochemical properties of the filter media. The removal efficiency of slow sand filtration is influenced by the degree of biofilm development (filter maturity). Biologically immature slow sand filters were found to be inefficient in the removal of microorganisms (Schuler et al., 1991). On the other hand, excessive biofilm accumulation on the filter media may reduce pore volume, increase frictional resistance, and affect removal efficiency. Accumulation of organic and inorganic materials (e.g., metal ions, sulfate, humic acids) in the biofilm leads to "ripening" of filter sand (Galvin, 1992). The degree of filter maturation was affected by backwashing conditions (Chipps et al., 1995). The precise effects of biofilm formation on bacterial transport and filter properties are poorly understood.

Mechanism of Biofilm Formation

Microbial adhesion to surfaces is a time-dependent process in which biogrowth increases as contact time increases (Zobell, 1943). Mechanisms of microbial transport to the supporting surfaces include Brownian motion and diffusion (due to the random bombardment of the medium surface by fluid particles), electrostatic interaction, interception, inertial impaction, and gravity sedimentation (Tien, 1989). Once in contact with the surface, microorganisms produce extracellular, polymeric material described as polysaccharide glycocalyx (Costerton et al., 1981). The extracellular material contains thin thread-like fibers referred to as fimbriae that extend from the cell wall to the contact surface (Sasahara & Zottola, 1993). The exopolymer matrix provides a "shelter" against the effect of toxic or inhibitory substances.

The process of biofilm formation may be characterized as involving three distinct steps: initiation, consolidation, and colonization (Zottola, 1994). Initially, microorganisms come into contact with and weakly adhere to the substrata by electrostatic attraction and van der Waals forces. This initial attachment process is reversible; cells may be easily removed by kinetic or mechanical forces such as shear force. Subsequently, the microorganisms produce extracellular, polymeric material that physically bonds the cells to specific sites of the surface by means of polymer bridges. This consolidation process is considered irreversible. Finally, the attached microorganisms multiply and colonize localized areas of the surface, forming microcolonies sometimes referred to as "patchy biofilms" (Tijhuis et al., 1994). As the microcolonies grow and spread over the surface, a film-like structure called "biofilm" eventually develops at the liquid-solid interface.

Others have argued (e.g., Characklis, 1984) for a 5-stage biofilm formation process, which includes (1) transport of nutrients, inorganic and organic particulate to the solid surface; (2) development of a "conditioning film" by adsorption of inorganic or organic nutrients; (3) attachment of microbial cells to the conditioned surface; (4) microbial metabolism, growth and decay within the biofilm; and (5) cell disruption and detachment from the biofilm. Biofilm growth is more strongly promoted by the adsorption of organic polymers (including polysaccharides and proteins) than of nutrients at the solid surface (Characklis, 1973). The effect of "conditioning films" on adsorption may vary, depending in part on the nature of the attaching surfaces (Schneider & Marshall, 1994). I propose that biofilm formation involves sequential steps illustrated in Figure 2-1.

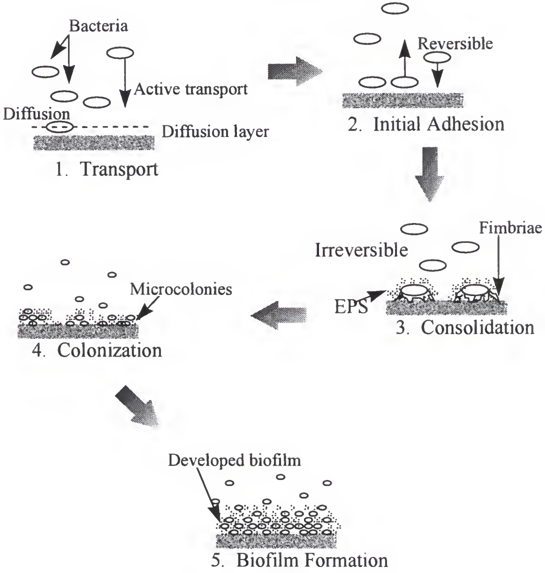

Figure 2-1. Schematic representation of sequential steps of biofilm formation.

Morphological and biochemical evidence suggest that bacteria are the primary colonizers in the process of biofilm formation on both artificial and natural substrata

(Burkholder & Wetzel, 1989; Liu et al., 1993). Depending on the characteristics of the fluid media and environmental conditions, other microorganisms (protozoa, diatoms, rotifers, fungi, algae, and worms) may become involved, although to a significantly lesser extent, during late stages of biofilm development (Liu et al., 1993).

Factors in Biofilm Accumulation

Bacterial adsorption to surfaces is a kinetic process and a steady-state can usually be reached (Kinoshita et al., 1993). The speed at which biofilm accumulates on a surface will depend on rates of microbial attachment, growth, decay, and detachment (Lewandowski et al., 1993; Rittmann, 1990). The net balance of these processes also determines the amount of biofilm accumulation at steady state. The time scale over which biofilm development is observed ranges from a few days in nutrient-supplemented water (Cunningham et al., 1991) to months in tap water (Pedersen, 1990). In the following, various factors affecting biofilm accumulation are described.

Substratum Material

Wolfaardt and Cloete (1992) found that the highest colonization rates occurred on stainless steel, followed by aluminum and glass. Biomass loss due to fluid shear in an activated carbon column was approximately twice that of sand or coal columns (Lowry & Burkhead, 1980). The rate of microbial adsorption was positively correlated with surface free energy and hydrophobicity, which were related to surface roughness of the substrata, in colonization of *Pseudomonas aeruginosa* and *P. fluorescens* on stainless steel, copper, silicon and glass surfaces (Mueller et al., 1992). Small, rough particles best facilitated biofilm formation in an air-lift suspension reactor (Heijnen et al., 1992). The probability of desorption decreased with increasing surface free energy and surface roughness. Chang and Rittmann (1988) found that an irregular (rough) granular activated carbon (GAC) surface provided better protection for initial biofilm accumulation than did a spherical (smooth) surface, due to difference in shear loss. Using pure cultures of *Pseudomonas putida*, Shreve et al. (1991) found that biofilm accumulation was greatest on charged surfaces [SiO_2 (-), Al_2O_3 (+), basic ion-exchange resin (+), nitrocellulose (-), derivatized nylon (+), borosilicate (+), cellulose ester (+)], intermediate on uncharged, hydrophobic surfaces (polystyrene, Teflon, polyvinylidene difluoride), and least on uncharged, hydrophilic surfaces (acrylic copolymer, modified polyvinylidene difluoride).

Surface Properties of Bacteria

Surface charge and hydrophobicity of bacteria are two important parameters affecting bacterial adsorption to solid surfaces (Bales et al., 1991). Surface charge is typically estimated by measuring the electrokinetic potential (zeta potential ζ) at the shear plane of the electrical double layer (Stern, 1924, refer to Rosen, 1989), because direct measurement of the true surface charge potential is practically impossible (Rosen, 1989). Since bacteria are treated as living colloidal systems (Marshall, 1976), zeta potentials of biofilm bacteria may be measured with bacterial suspensions by microelectrophoresis (e.g., Van der Mei et al., 1993). Hydrophobicity can be measured by the water contact angle method (Busscher et al., 1984; van Loosdrecht et al., 1987) or microbial adhesion to hydrocarbons (MATH) (Lichtenberg et al., 1985; Van der Mei et al., 1987). The main advantages for using MATH are its ease and simplicity. Reproducible MATH results can be obtained when experimental conditions (ionic strength, buffer pH, oil:water volume ratio, test tube dimensions, etc.) are controlled (Gibbons & Etherden, 1983). Zeta potential has been observed to increase with decreasing hydrophobicity (van Loosdrecht et al., 1987).

Surface Modification of Filter Media

Since bacteria assume a negative surface in most natural environments, rendering the adsorbent surfaces more electropositive would enhance adsorption by reducing electrostatic repulsion and increasing sticking efficiency (α) (McCaulou et al., 1994). Coating the adsorbent surfaces with multivalent ions, such as Al^{3+}, Ca^{2+} and Fe^{3+}, can change the magnitude and even the sign of zeta potential. Sand amended with 30% red mud (neutralized with 5% gypsum), which is rich in iron oxides, significantly increased removal efficiency for both *E. coli* and *Salmonella adelaide* (Ho et al., 1991). Enhanced bacterial adsorption to surfaces coated with oxyhydroxides (which are rich in ion, aluminum, and manganese) was also observed (Scholl & Harvey, 1992). Viral adsorption to hydrophobic polymer-coated silica was shown to be markedly higher than to hydrophilic (unmodified) surfaces (Bales et al., 1991). Batch adsorption has been used as a rapid screening procedure to evaluate microbial adsorption to surface-modified or unmodified porous media (e.g., Farrah & Preston, 1991; Mills et al., 1994; Scholl & Harvey, 1992). The effect of surface properties on filter coefficients can be estimated using a steady-state colloid filtration model (Harvey & Garabedian, 1991).

Solution Conditions

The magnitude of hydrophobic or electrostatic effects on adsorption is influenced by solution variables such as ionic strength and pH (Fontes et al., 1991). Since natural surfaces are usually negatively charged, adsorption increases with increasing ionic strength (Mills et al., 1994; Sharma et al., 1985) to a point where the electrical double layer is compressed and van der Waals attractive forces overcome the repulsive forces (Scholl & Harvey, 1992; Stenkamp & Benjamin, 1991). The opposite is true for positively-charged surfaces (Scholl & Harvey, 1992; Zerda et al., 1985). Strong pH dependencies were found in batch and column experiments using aquifer sediment (sand) and indigenous bacteria concentrated from contaminated groundwater samples (Scholl & Harvey, 1992). Generally speaking, bacterial adsorption decreases with increasing pH (Scholl & Harvey, 1992; Scholl et al., 1990). The effect of pH-dependent surface charge was also demonstrated in viral adsorption to charge-modified silica (Zerda et al., 1985). The effect of pH may be stronger on microbial surface properties than on those of the adsorbent. For example, altering solution pH did not result in detectable change in electrophoretic mobility of silica used in packed-beds (Bales et al., 1991), but had a modest but statistically significant effect on bacterial transport (Kinoshita et al., 1993).

Conditioning Film

The organic and inorganic substances in natural waters, if adsorbed to substrata, may generate a new interface called "conditioning film" with altered physicochemical properties (Schneider et al., 1994). Dissolved organic matter, which is thought to possess some of the same functional groups as possessed by bacteria, adsorbs to natural surfaces similarly as bacteria and thus could compete with bacteria for surface sites (Scholl & Harvey, 1992). Proteins are strongly adsorbed to hydrophobic surfaces, while adsorption to hydrophilic surfaces is dominated by electrostatic interactions (Stuart et al., 1991). Formation of "conditioning films" is influenced by the degree of water contamination (Scholl & Harvey, 1992).

Dilution Rate

Low hydraulic retention time, or high dilution rate, favors biofilm formation (Sreekrishnan et al., 1991). In air-lift suspension reactor, the dilution rate must be greater than the maximum specific growth rate of the suspended bacteria for complete biofilm formation to occur (Tijhuis et al., 1992). The effect of substrate surface loading rate is on the amount of biomass; a higher surface load leads to a thicker biofilm (Tijhuis et al., 1994).

Shear Stress

On stainless steel, aluminum and glass surfaces, colonization rates decreased with increasing flow velocity, with the highest colonization rates being associated with conditions of zero flow (Wolfaardt & Cloete, 1992). The influence of shear due to particle-particle interactions diminishes after complete development of biofilms (Heijnen et al., 1992). Shear stress also affects biofilm morphology and structure. Weak shear stress (low flow velocities) usually leads to rougher, more heterogeneous biofilms (Lewandowski et al., 1993). More rigid and homogeneous biofilms develop in the presence of strong shear stress (high flow velocities) (Characklis, 1973).

Biofilm Structure

In biofilms, microbial cells are immobilized next to each other and embedded in a gel-like matrix composed of exopolymers (EPS) (Decho, 1990), forming well organized, physiologically cooperative consortia with sorbent properties (Flemming, 1993). The biofilm matrix represents a diffusion barrier for mass transfer and therefore affects transport of substrates such as oxygen and nutrients. Confocal scanning laser (CSL) microscopy indicated that biofilm bacteria grow in EPS matrix-enclosed microcolonies interspersed with less dense regions represented by highly permeable water channels (Caldwell et al., 1992; Lawrence et al., 1991) that may facilitate the transport of substrates such as oxygen into the biofilm (Costerton et al., 1994). Direct examinations using chemical probes such as fluorochrome-coupled dextrans of various molecular weights showed that dextrans having sizes on the order of 2,000 kDa readily penetrated the water channels (refer to Costerton et al., 1994). Thus, small molecules such as glucose can be readily transported into biofilms through the water channels.

Biofilms are heterogeneous in space and time (Flemming, 1993). The competition for substrate within biofilms results in structural stratification with non-uniform spatial distributions of biofilm properties (Zhang et al., 1995). Biofilm density increased markedly with increasing depth, while the opposite was true for porosity, specific surface area, and mean pore radius (Zhang & Bishop, 1994). Consequently, effective diffusivity was found to decrease appreciably with increasing depth. Densities in the bottom layers were 5-10 times higher than those in the top layers; the porosities decreased from 84-93% in the top layers to 58-67% in the bottom layers; and the mean pore radius decreased from 1.7-2.7 μm in the top layers to 0.3-0.4 μm in the bottom layers. The ratio of living cells to total biomass changed from 72-91% in the top layers to 31-39% in the bottom layers. Availability of oxygen decreases vertically with increasing depth. Bottom layers may be devoid of oxygen (Hamilton, 1987), providing a habitat for anaerobic organisms even in otherwise aerobic environments (Hamilton & Characklis, 1989).

Morphological and topographic data of biofilms are derived from micrographic image analysis. Although light microscopy may be used for such purposes (e.g., Zahid &

Ganczarczyk, 1994), scanning electron microscopy (SEM) has increasingly become the method of choice for its high resolution. A minus for using SEM is the extensive sample preparation required for biological material, including fixation, dehydration and drying, because it operates at high vacuum. Furthermore, biofilm samples must be coated with a conductive thin metal film (usually of silver or gold) to be viewed by SEM, because nonconductive samples such as biofilms build up local concentrations of electrons, referred to as "charging," that prevent the formation of usable images (Little et al., 1991). Such sample preparation procedures could damage the cells, alter the properties of the biofilm samples, and compromise data integrity.

Energy dispersive X-ray spectroscopy (EDS), which is used to determine the elemental composition of biofilms, can be made part of a SEM procedure (Little et al., 1991). This step is completed prior to the deposition of metal coating. A new development in SEM technology is the so-called "environmental scanning electron microscopy" (ESEM), which utilizes a secondary electron detector capable of obtaining images of high resolution at relatively high pressures (in the range of 0.1-20 torr) (Little et al., 1991). At relatively high pressures, specimen charging is dissipated into the gaseous environment of the sample chamber, thus allowing direct observation of uncoated, nonconductive biological samples. Since no metal coating is required, acquisition of EDS data can be done simultaneously. Another advantage of ESEM is that wet samples can be observed without pre-drying by using water vapor as the specimen environment. ESEM not only simplifies the procedure, but increases data accuracy as well by minimizing the artifacts introduced during extensive sample preparation required for SEM (Little et al., 1991).

The porosity of biofilms is determined indirectly by estimating the portion of a biofilm that is occupied by water outside of the cells (Wanner, 1989). The specific surface area of biofilms can be determined by dye adsorption test (Smith & Coackley, 1983). There has been interest in measuring biofilm activity, which directly effects biofilm development (Liu et al., 1993), by use of certain biochemical indicators such as enzyme activity (Roth & Lemmer, 1994), electron transport system (ETS) activity (Blenkinsopp & Lock 1990; Trevors et al., 1982), and ATP content (Gikas & Livingston, 1993; Nouvion et al., 1987). Microsensor techniques have also been used to study biofilm development *in situ* (Nivens et al., 1993).

Extracellular Polymers Associated with Biofilms

Since bacteria in biofilms are enveloped by a matrix of extracellular polymers or exopolymers (EPS), the properties of this matrix largely dictate the physical chemistry of the biofilm. The polymeric matrix is a polysaccharide complex with hydrophobic, hydroxyl, carboxyl, and phosphate moieties (Marshall, 1985), which also includes proteins and phospholipids to a minor extent (Sutherland, 1984). This matrix can account for as much as 50-90% of the total organic carbon content in the biofilm (Bakke et al., 1984). The EPS mediates interaction of bacteria with substrata leading to irreversible adhesion and is key to the structural integrity of biofilms (Decho, 1990; Neu & Marshall, 1990).

Exopolysaccharides consist of specific polysaccharides and nonspecific polysaccharides (Kenne & Lindberg, 1983). Specific polysaccharides are those characteristic of individual bacterial strains with unique antigenic properties. This group of polysaccharides is typically composed of such common sugars as glucose, galactose and mannose, as well as methylpentoses (e.g., rhamnose), amino sugars (e.g., N-acetylglucosamine and N-acetylgalactosamine) and uronic acids (e.g., glucuronic acid and galacturonic acid) (Christensen & Characklis, 1990; Sutherland, 1977). Structurally, specific polysaccharides are typically composed of oligosaccharide repeating units of 2-9 monosaccharides. Additionally, organic substituents such as pyruvate and acetate are commonly found in bacteria. Inorganic substituents such as sulfate are less common in bacteria. Nonspecific polysaccharides are structurally different and generally simpler than specific polysaccharides. Many of them are homopolysaccharides, containing only one monomer, such as glucans (composed solely of glucose), frucans (composed exclusively of fructose), and cellulose (similar to β-1,4-linked glucan cellulose of plant origin) (Sutherland, 1977).

Bacterial surfaces range from highly hydrophobic to highly hydrophilic, depending on the species and environmental conditions. Table 2-1 lists reported hydrophobicity values for some bacteria. Polysaccharides are usually regarded as hydrophilic, due to the abundance of hydroxyl groups found in carbohydrates. Proteins may contain amino acids with hydrophobic side groups. The type and relative quantity of polysaccharides, proteins and possibly lipids in the EPS may determine the hydrophobicity of biofilms (Gilbert et al., 1993). Polysaccharides can exhibit amphophilic properties (Symes, 1982). Hydrophobic properties may be present either due to hydrophobic groups such as methyl groups and acetyl groups, or to "polarized" arrangements of the hydroxyl groups (hydrophilic) and the ring protons (hydrophobic). Thus, hydrophobicity may be influenced by the conformation of the carbon ring as well as the type of sugars. Lipopolysaccharides and teichoic acids, which are components of the cell wall but are

Table 2-1. Hydrophobicity of bacteria as measured by contact angle or MATH

Bacteria	Hydrocarbon	Aqueous medium	pH	Contact angle θ (°)	MATH (% removal)***	References
Alcaligenes sp.		P-buffered saline	7.2	23		Loosdrecht et al., 1990
Arthrobacter spp.		P-buffered saline	7.2	38-60		Loosdrecht et al., 1990
Azospirillum brasilense	Hexane	PUM buffer*	7.1		43-67	Achouak et al., 1994
Azospirillum lipoferum	Hexane	PUM buffer	7.1		14	Achouak et al., 1994
Bacillus circulans	Hexane	PUM buffer	7.1		21	Achouak et al., 1994
Bacillus polymyxa	Hexane	PUM buffer	7.1		19-42	Achouak et al., 1994
Enterobacter agglomerans	Hexane	PUM buffer	7.1		10	Achouak et al., 1994
Escherichia coli		P-buffered saline	7.2	16		Loosdrecht et al., 1990
Pseudomonas aeruginosa	Hexadecane	P-buffer**			99	Mueller et al., 1992
	Octane	P-buffer			95	Mueller et al., 1992
		P-buffered saline	7.2	25		Loosdrecht et al., 1990
Pseudomonas cepacia	Hexane	PUM buffer	7.1		77	Achouak et al., 1994
Pseudomonas fluorescens	Hexadecane	P-buffer			80	Mueller et al., 1992
	Octane	P-buffer			54	Mueller et al., 1992
Rahnella aquatilis	Hexane	PUM buffer	7.1		16-50	Achouak et al., 1994

* PUM buffer = 22.2 g $K_2HPO_4 \cdot H_2O$ + 7.3 g KH_2PO_4 + 1.8 g urea + 0.2 g $MgSO_4 \cdot 7H_2O$ per liter; 0.5 mL hexane was added to 1.2 mL of washed cells suspended in PUM (pH 7.1); total vortexing time = 120 s.

** Rosenberg et al., 1980; Rosenberg, 1984.

***Increasing % removal indicates increasing hydrophobicity.

generally not considered as extracellular polysaccharides, may also play a role in determining the physicochemical properties of biofilms, at least for cells in the top (biofilm surface) layer.

Biofilms exhibit ion exchange capacity due to the presence of charged groups associated with EPS and cell wall components, which can bind oppositely charged colloids such as heavy metals (Geesey, 1982). Differences were found between pH in the bulk solution and in the biofilm due to accumulation of organic acids entrapped in the EPS matrix (Flora et al., 1993), contributing to sorbent properties of the EPS matrix. Teichoic acid residues characteristic of Gram-positive bacteria such as *Bacillus* have ion-exchange functions. Similarly, Gram-negative bacteria, although lacking the ability to produce teichoic acids, possess acidic polysaccharide components (e.g., guluronic acid and mannuronic acid) with uronic acid residues that also exhibit ion-exchange capacity. Ion-exchange functions of biofilms may include general ion-binding and selective binding of cations.

In general, EPS are capable of binding metallic cations as counterions through ionic interactions. Teichoic acids can maintain high levels of divalent cations, but exhibit especially strong selectivity for binding Mg^{+2} over Na^+, K^+, or Ca^{+2} (Lambert et al., 1975). Mannuronic acid residues are not selective toward divalent cations. However, guluronic acid residues are selective for Ca^{+2} in relation to Mg^{+2} and for Sr^{+2} in relation to Mg^{+2}, but less so for Sr^{+2} in relation to Ca^{+2}. Both uronic acids are selective for Cu^{+2} in relation to Ca^{+2}, which is a characteristic feature of all carboxylate-containing polymers and polyphosphate, but not of sulfated polysaccharides (Sutherland, 1977). Such selectivity provides a basis for selective removal of toxic metals and other colloids.

Besides ion exchange, polysaccharides can bind metallic ions by forming complexes through chelation and coordination.

The presence of ionizable functional groups (such as carboxyl, hydroxyl and amino groups, and phosphate) on bacterial surfaces is responsible for bacterial surface charge (James, 1957). A predominance of hydroxyl groups associated with polysaccharides relative to amino groups would tend to give biofilms an overall negative surface charge. The negative charge may be further strengthened by carboxyl groups associated with organic acids entrapped in the EPS matrix due to bacterial metabolism. In a study of interactions of rhizobacteria with rice roots, electrophoretic potential values indicated that all test bacteria exhibited a negative surface charge (Achouak et al., 1994). Harden and Harris (1953) determined the isoelectric points (IEP) for 31 bacterial species and also gave IEP values for 27 bacterial species (some overlapping) determined previously by other investigators. Accordingly, isoelectric points for most bacteria seem to be in the range of 2 to 4, implying a negative surface charge in normal pH range found in natural aquatic environments. Recently reported zeta potential values for some bacteria are listed in Table 2-2.

Interactions of Bacteria and Minerals

Bacterial adhesion to minerals may take place even when both surfaces are negatively charged, as shown in a study of bacterial adhesion to apatite minerals, $Ca_5(PO_4)_3OH$ (hydroxyapatite), $Ca_{10}(PO_4)_6F_2$ (fluorapatite) and $CaHPO_4 \cdot 2H_2O$ (brushite) (Yelloji Rao et al., 1993). Exposure to the apatite minerals in solution caused the zeta potential of *Actinomyces naeslundii* and *Streptomyces sanguis* cells to become less electronegative. The mineral fines after exposure to bacteria exhibited zeta potential

Table 2-2. Zeta potentials of bacteria

Bacterium	Medium	pH	ζ (mV)	IEP (pH)	References
Actinomyces naeslundii	Water	7.0	~ -28	3.7	Yelloji Rao et al., 1993
Azospirillum brasilense	0.01 M KCl	7.0	-14.7 to -15.9	2.0	Achouak et al., 1994
Azospirillum lipoferum	0.01 M KCl	7.0	-25.0	2.0	Achouak et al., 1994
Bacillus circulans	0.01 M KCl	7.0	-18.3	< 2.0	Achouak et al., 1994
Bacillus polymyxa	0.01 M KCl	7.0	-2.0 to -12.6	< 2.0 to 4.5	Achouak et al., 1994
Enterobacter agglomerans	0.01 M KCl	7.0	-35.0	< 2.0	Achouak et al., 1994
Pseudomonas aeruginosa	P-buffered saline	7.0	-2.9 to -11.4		Nicholov et al., 1993
Pseudomonas cepacia	0.01 M KCl	7.0	-22.0	< 2.0	Achouak et al., 1994
Rahnella aquatilis	0.01 M KCl	7.0	-24.5 to -38.0	2.0	Achouak et al., 1994
Scillatoria spp.*	Water	6.57-6.84	-12.3 to -69.2		Bender et al., 1994
Streptococcus sanguis	Water	7.0	~ -34	3.2	Yelloji Rao et al., 1993
Streptococcus thermophilus	P-buffer		20.0 to -55.0	3.0 - 4.6	Van der Mei et al., 1993
Streptomyces clavuligerus	0.5% ethanol	2.0 - 11.0		< 2.0	Matis & Zouboulis, 1994
Streptomyces griseus	0.5% ethanol	2.0 - 11.0		2.4	Matis & Zouboulis, 1994
Sulfolobus acidocaldarius	Mineral salts**	7.0	-2.0	< 1.0	Vitaya & Toda, 1991

* Biofilm composed of mixed populations of photosynthetic and heterotrophic bacteria dominated by *Scillatoria* spp.

** Containing 0.2 g NaCl, 0.3 g KH_2PO_4, 1.3 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, and 0.05 g $CaCl_2 \cdot H_2O$ per liter of distilled water.

values between those of the mineral and of the bacteria, although closer to that of the mineral, as revealed by streaming potential measurement. Mineral-bacteria aggregates exhibited zeta potentials markedly more negative than the normal values of either the minerals or the bacteria. Less negative zeta potentials were obtained after conditioning (washing) the bacteria in distilled water. However, zeta potential changes did not affect bacterial adhesion, suggesting that electrostatic interactions were not the primary factors determining adhesion in this case.

Similarly, an increase in net negative charge was observed on kaolin clay and quartz particles after interacting with bacteria (*Bacillus cereus*) (Ghalambor et al., 1994). This may be due to the release of bacterial extracellular components such as polysaccharides to the particle surfaces. Surface property changes due to interaction are influenced by environmental conditions. For example, a reduction in negative charge density of polysaccharides and clay- polysaccharide particles was observed under reduced pH levels (Kanaani et al., 1992). Polymeric conformational changes induced by variation in ionic strength or pH may play a role in bacterial adsorption to clay surfaces. Quantitative, compositional, or conformational changes of exopolysaccharides may partially explain the "averaging" and "additive" effects of negative charges between the interacting surfaces in the case of mineral-bacteria interactions (Yelloji Rao et al., 1993).

Interactions of Bacteria and other Colloids with Biofilms

Microbial transport through porous media is modulated by surface-to-surface interactions between microorganisms and solid matrices (Camper et al., 1993; Gannon et al., 1991; Schneider et al., 1994; Scholl et al., 1990; Wan et al., 1994). Bacteria in aqueous media exhibit colloidal characteristics (Marshall, 1976). Transport models have been developed based on the colloid filtration theory (Yao et al., 1971). The presence of a biofilm can be expected to alter the physical, chemical, and physicochemical properties of the attaching surface. Initial colonization of surfaces increases surface roughness, which promotes biofilm formation (Lewandowski et al., 1993). According to Bouwer (1987), increased surface roughness can influence particle transport and microbial attachment rates by enhancing convective mass transport, providing shelter from shear forces, and increasing surface area for attachment. The purifying effect of slow sand filters is primarily due to the formation of biofilms on the sand grains (Duncan, 1988).

In a latex tracer (with a nominal diameter of 1 μm) study (Drury et al., 1993), fluorescent latex microbeads penetrated the *Pseudomonas aeruginosa* biofilm and remained in it much longer than predicted by a model of advective displacement due to cell growth. The microbeads added to the bulk fluid became distributed throughout the biofilm depth, some being able to penetrate to the substrata within 24 h. The biofilms were rough and had a mean thickness of approximately 34 μm with a maximum thickness of approximately 35 μm at maturity. The bead release rates corresponded to first order time coefficients of 0.01-0.03 h^{-1} and were approximately an order of magnitude less than the predicted time scale of advective transport, which is the experimentally measured specific cellular growth rate of 0.15 h^{-1}. The rough nature of the biofilm was speculated as the reason for the potent bead penetration and unexpectedly low advective displacement velocity of particles in the biofilm.

Some experimental evidence suggests that biofilm accumulation increases colloid cohesion coefficient (α) (also referred to as "sticking efficiency"). For example, moderate biofilm accumulation achieved with low influent chemical oxygen demand

(COD) loading caused α to increase from 0 on clean surface to 0.04 on biofilm-covered surface in an anaerobic, fluidized bed biofilm reactor using granular activated carbon (GAC) as the supporting media (Sprouse & Rittmann, 1990). However, greater biofilm accumulation due to higher influent COD loading dramatically increased α and therefore improved capture (removal) of organic colloids (Rittmann & Wirtel, 1991). The steady-state α value obtained with GAC colonized with 1.74 mg cellular carbohydrate per gram of dry carbon was 0.9 compared to $\alpha = 0.21$ for GAC with 0.134 mg cellular carbohydrate per gram of dry carbon. The increased α probably resulted from the accumulation of extracellular polymers (such as polysaccharides) that form the gelatinous matrix within which bacterial cells are entrapped (Bryers, 1988).

Biofilm formation alters surface geometry and increases surface area for deposition of solids by gravity or interception of particles in the bulk flow (Bouwer, 1987). It was reported that bacterial cells attach to biofilms at higher rates than to clean glass surface (Banks & Bryers, 1992). Similarly, the fraction of attached latex microbeads was found proportional to biofilm quantity and thickness with a rotating annular reactor under turbulent flow conditions (Drury et al., 1993). There is a direct relationship between surface geometry and surface area. The existence of pores in biofilms increases surface area for contact and attachment and provides a possible mechanism for biofilms to envelop bacterial cells and other colloidal particles. Particles attached within pores may be protected from hydrodynamic shear stress and are therefore less likely to detach than particles not in pores.

Biofilm formation was found to cause reduction of the saturated hydraulic conductivity, K_s, of natural sand (Vandevivere & Baveye, 1992). Scanning electron micrographic analysis showed that the ability of an obligately aerobic bacterial strain, *Arthrobacter* sp., to clog permeameters filled with fine sand was largely due to the formation of aggregates of cells that plug the pores. Production of exopolymers (EPS) was absent under O_2- or glucose-limited conditions. However, the cells produced EPS under N-limited conditions.

Removal of Biofilm

A critical step in biofilm analysis is the removal of fixed biomass from its substrata (Lazarova & Manem, 1995; Pierzo et al., 1994). Most studies on biofilm formation have been conducted on surfaces of such materials as cast iron cylinders (Donlan et al., 1994), stainless steel coupons (Uzcategui et al., 1991), mini glass slides (Liu et al., 1993), and polycarbonate membranes (Drury et al., 1993). Few studies have dealt with the removal of biofilm mass from surfaces of natural granular materials such as sand. Some biofilm development data have been obtained by monitoring the influent and effluent biomass concentrations of COD or bacterial numbers (e.g., Sprouse & Rittmann, 1990).

Table 2-3 lists some commonly used removal methods and their relative advantages and disadvantages. Removal methods may be categorized into physical and chemical methods, the use of which depends on the purpose of study and nature of supporting media (substrata). For example, physical methods such as scraping (Donlan et al., 1994; Geesey et al., 1978; Lechevallier et al., 1987) are limited by the size and shape of substrata. Scraping may be useful for such surfaces as stone (Geesey et al., 1978), stainless steel coupons (Uzcategui et al., 1991), cast iron cylinders (Donlan et al., 1994), and glass slides, but is not applicable in filter studies involving granular media such as fine sand.

Table 2-3. Methods used for removal of biofilms from substrata

Method	Solvent	Assay	Comments	References
Physical				
Scraping	Water	*	Limited by size & shape of substrata	Donlan et al., 1994
Shaking	Water	*	Incomplete removal	Sreekrishnan et al., 1991
Vortexing	Water	*	Incomplete removal	Ehlinger et al., 1987
Sonication	Water	*	Incomplete removal	Pierzo et al., 1994; Zips et al., 1990
Homogenization	Water	*	Incomplete removal	Cunningham et al., 1991
Chemical				
Acid	Phenol/H_2SO_4	Polysaccharides	Dissolves coating on substrata	Liu et al., 1993
Base	NaOH	Proteins	Dissolves coating on substrata	
Organic solvent	SDS or urea	Proteins	Increase recovery	Current Protocols in Molecular Biology
	H_2O/Teepol	Enumeration	Increase removal	Pell & Nyberg, 1989
	Chloroform-methanol	Phospholipids	Complexity of separations	Findlay et al., 1989
Other	Sodium pyrophosphate	Proteins	Incomplete removal	Eighmy et al., 1992

* Assays not specific to removal method.

Physical removal by shaking (Eighmy et al., 1992; Sreekrishnan et al., 1991), vortexing (Ehlinger et al., 1987), sonication (Pierzo et al., 1994; Zips et al., 1990) and homogenization (Cunningham et al., 1991) has been used to produce suspension samples or in conjunction with chemical removal. The microorganisms are released to and suspended in a buffered or unbuffered aqueous solvent such as tap water (Sreekrishnan et al., 1991) or distilled water (Cunningham et al., 1991) for analysis. In conjunction with mechanical treatments, chemicals such as Teepol (Pell & Nyberg, 1989), Triton and Tween (Yoon & Rosson, 1990) and sodium pyrophosphate (Eighmy et al., 1992) may be added to the aqueous solvent to enhance removal.

Chemical removal methods such as extraction and digestion are used in conjunction with biochemical analyses, such as polysaccharide assay (in phenol-H_2SO_4) (Dubois et al., 1956; Kuo & Bitton, 1995), protein assay (in NaOH solution), and phospholipid assay (in chloroform-methanol-phosphate buffer) (Findlay et al., 1989).The removal procedure may introduce problems or artifacts, depending on the nature of subsequent analysis. Physical methods such as sonication often result in incomplete removal (Pierzo et al., 1994), leading to underestimation of biofilm mass. Chemicals used in conjunction with extraction or digestion procedures may dissolve coatings on the supporting surfaces, causing interference with subsequent analysis. Kuo and Bitton (1995) found that acid used in conjunction with polysaccharide assay involving $Al(OH)_3$-coated sand led to high blanks. Use of COD procedure suffered similar interference. Bacterial enumeration requires great care to avoid cell lysis or damage. Vigorous physical removal, such as homogenization (blending), or chemicals used in conjunction with physical removal can lyse cells and thus interfere with plate counts or direct microscopic counts.

Quantitative Estimation of Biofilm Mass

Biofilm mass is approximated by estimation of wet or dry weights (Pedersen, 1982), ash-free weights (Galvin, 1992), biofilm thickness (Bakke & Olsson, 1986), total organic hydrocarbon (TOC) (Harris & Kell, 1985), chemical oxygen demand (COD) (Characklis et al., 1982; Rittmann et al., 1986), microbial enumeration (Pedersen, 1990), or biochemical assay (Lazarova & Manem, 1995). Commonly used methods for biomass estimation are listed in Table 2-4. The ease and precision of many biochemical assays make them good candidates for monitoring biofilm development by measuring specific components of biofilms. Key information about the methods used or considered for the present study are described in the following.

Polysaccharide Assay

The phenol-H_2SO_4 method (Dubois et al., 1956) or its variations (e.g., Liu et al., 1992, 1993) is frequently used to measure polysaccharide quantity. Biofilm is removed from substrata and subjected to acid hydrolysis in H_2SO_4 to break polysaccharides into oligosaccharides. After incubation in phenol-H_2SO_4, the supernatant is used to obtain absorbance at 485 nm (Kuo & Bitton, 1995). The method is based on the reaction of carbohydrate reducing ends (ketones or aldehydes) with strong, nonoxidizing acid, such as H_2SO_4. The reaction yields hydroxymethyl furfural plus other by-products. Condensation between these activated aldehydes and phenolic compounds leads to the formation of colored compounds as a direct function of polysaccharide concentration (Characklis, 1990).

Table 2-4. Comparison of methods used for estimation of biofilm mass

Method	Technique	Comments	References
Enumeration	Plate counts	Viable/culturable cells only	APHA, 1992; Prakasam & Dondero, 1967
	MPN	Viable/culturable cells only	Alexander, 1982; APHA, 1992; Pell & Nyberg, 1989
	Epifluorescence microscopy	All cells, sensitive, background fluorescence	APHA, 1992; Kemp et al., 1993; Kepner & Pratt, 1994; Sieracki & Viles, 1992
Total biomass	Wet/dry weights	Simple, nonspecific	Characklis, 1990; Pedersen, 1982
	Ash-free dry weights	Simple, nonspecific	Galvin, 1992
	TOC	Sensitive, interference from organic coatings	Bakke et al., 1984; Harris & Kell, 1985; Krambeck et al., 1981
	COD	Precision, interference from metal coatings	Characklis et al., 1982; Rittmann et al., 1986
Biochemical assay	Polysaccharides	Acid can dissolve coatings on media	Liu et al., 1992, 1993
	Exopolysaccharides	Indirect, overestimation	Dubois et al., 1956; Ford et al., 1991; Horan & Eccles, 1986
	Lipopolysaccharides	Underestimation, cost	Watson & Hobbie, 1979
	Phospholipids	Sensitive, poor indicator for dead cells	Bligh & Dyer, 1959; Findlay et al., 1989; Freeman & Lock, 1995; White et al., 1979a,b,c; Zhang & Bishop, 1994
	Proteins	Interference from coatings	Bradford, 1976; Davis, 1988; Eighmy et al., 1992; Hanson & Phillips, 1981; Lowry et al., 1951; McKinley & Vestal, 1985
	DNA (fluorometry)	Spectral interference, poor reproducibility	Ceasarone et al., 1979; McCoy & Olson, 1985; Paul & Myers, 1982; Tharakan & Chau, 1984
Other	Total N	Digestion required	APHA, 1992
	Total P	Digestion required	APHA, 1992

Phospholipid Assay

Phospholipids are considered an indicator of viable biomass (Vestal & White, 1989) since they are not stored but turned over in nature, with a measured half-life of hours to a few days (Zhang & Bishop, 1994). The phospholipid content of a given species is relatively constant, and colorimetric determination is relatively simple, sensitive and reproducible (Lazarova & Manem, 1995). To recover phospholipids, biofilm samples are homogenized and extracted for 2-24 h in a solvent consisting of chloroform, methanol, and 50 mM phosphate buffer (pH 7.4) in a ratio of 1:2:0.8 so that the volume of chloroform in milliliters is 7 or more times than the fresh weight of the sample in grams (Findlay et al., 1989). The chloroform portion can be filtered to remove debris and inorganic solids (this step may be eliminated) and then vacuum-dried (Findlay et al., 1989) or oven- dried at 103 °C (Zhang & Bishop, 1994). Phosphate is liberated from the recovered lipids by potassium persulfate digestion (Findlay et al., 1989). The phosphate released by digestion is determined spectrophotometrically by the addition of ammonium molybdate and measurement of absorbance at 610 nm (Zhang & Bishop, 1994).

Protein Assay

Proteins account for approximately 50% of bacterial mass (dry weight). Total protein can be determined by colorimetric methods of Lowry (1951), Bradford (1976) or Clark and Switzer (1977). Lowry's method is recommended for the estimation of biofilm mass (Hanson & Phillips, 1981) because of its sensitivity and reproducibility (McKinley & Vestal, 1985). To recover protein, fresh samples of biofilm-coated sand are extracted in 0.1 N NaOH at room temperature for 10 min, then frozen at <-40 °C for 30 min, then thawed (in water bath) at 50 °C for 10 min. This freeze-thaw cycle promotes cell lysis and thus protein release. The resulting supernatant is used for protein analysis (Lowry's method). [Eighmy et al. (1992) used buffered 0.1% (w/v) sodium pyrophosphate solution (pH 7.0) for initial extraction prior to NaOH (1.0 N) digestion (at 90 °C for 10 min)]. Lowry's reagent (alkaline reagent) is added first, followed by the addition of Folic-Ciocalteau phenol reagent (Sigma Chemical Co., St. Louis, MO). After 30 min of color development, sample absorbance is measured at 750 nm. Bovine serum albumin is used to develop a standard curve. The alkaline reagent complexes with the peptide bonds and forms a purple-blue color when the Folic-Ciocalteau phenol reagent is added. Absorbance is read at an appropriate wavelength between 500 and 800 nm. The protein concentration is determined from the calibration (standard) curve.

Use of Nucleic Acid Stains to Tag Bacteria

Bacteria can be visualized and counted directly under an epifluorescence microscope when stained with fluorochromes. The bound dyes produce a fluorescent glow which allows cells below the resolution of light microscopy (generally < 1 μm) to be visualized and distinguished from other particles (Porter & Feig, 1980). Fluorochrome-mediated enumeration procedures are sensitive and specific, usually without the need for sample preparation. Both viable and dead cells can be accounted for. Acridine Orange (AO) and 4', 6-diamidino-2-phenylindole (DAPI) are the two most frequently used stains in epifluorescent direct count methods (Kepner & Pratt, 1994).

AO is similar to Ethidium Bromide (EB) in that they bind to both DNA and RNA by means of intercalation within the DNA double helix (Paul & Myers, 1982; Tharakan & Chau, 1984). Although AO stains nucleic acids preferentially, it can stain cytoplasm and even flagella (Sherr et al., 1993). AO fluoresces green when bound to DNA and orange red when bound to RNA (Hobbie et al., 1977). However, Bitton et al. (1993) found that the color of the fluorescing cells is dependent on the moisture level of the filter rather than the viability of the cells as previously thought (Hobbie et al., 1977). The percentage of cells fluorescing green can be increased by increasing the moisture level of the filter. This can be advantageous, since cells fluorescing green are more easily visualized than those fluorescing orange red (Bitton et al., 1993).

DAPI is a nonintercalating, DNA-specific stain (Coleman, 1980; Porter & Feig, 1980). The DNA-DAPI complex fluoresces bright blue at ≥390 nm when excited with light at 365 nm (Porter & Feig, 1980). DAPI binds preferentially with adenine-thymine (A+T)-rich regions of double-stranded DNA with a minimum requirement of 3 consecutive A-T base pairs (Kepner & Pratt, 1994). The structurally related Hoechst 33258 also binds preferentially with DNA regions which are rich in (A+T) (McCoy & Olson, 1985; Paul & Myers, 1982; Weisblum & Haenssler, 1974). Both can bind DNA in the major groove of the DNA double helix without intercalation, perhaps by hydrophobic interaction with the methyl of thymidine (Comings, 1975).

DAPI is superior to AO in terms of visualization and specificity, especially in the presence of high background fluorescence (Porter & Feig, 1980). AO is known as a nonspecific biopolymer that can indiscriminately stain like-sized biological and nonliving particles (Sieracki & Viles, 1992), such as clay, detritus, or colloids, some of which may also autofluoresce (Ghiorse & Balkwill, 1983; Yu et al., 1995). Complications often arise in AO-based enumeration procedures due to high background fluorescence (Porter & Feig, 1980). Characteristics of some commonly used fluorochromes are given in Table 2-5.

Table 2-5. Characteristics of selected fluorochromes

Fluorochrome*	Excitation wavelength	Emission wavelength	Target site	References
Acridine Orange	470-500	550-570	DNA, RNA	Sherr et al., 1993
	436, 490			Porter & Feig, 1980
	450-490	550		Sieracki & Viles, 1992
	470			Kepner & Pratt, 1994
DAPI	365	≥390	DNA	Porter & Feig, 1980
	350-380			Coleman, 1980
	355	450		Grimason et al., 1994
		390-400		Sherr et al., 1993
		470-480		Yu et al., 1995
Ethidium Bromide	519	619	DNA, RNA	Tharakan & Chau, 1984
	390-490	>510		Roser, 1980
FITC	470-490	500-520	Cytoplasm	Sherr et al., 1993
Hoechst 33258	≤356	≥415	DNA	McCoy & Olson, 1985
	350	450		Paul & Myers, 1982
Primulin	365	425	Cytoplasm	Sherr et al., 1993
Proflavin	470-490	500-520	Cytoplasm	Sherr et al., 1993

* Acridine orange = 3, 6-bis[dimethylamino]acridinium chloride; DAPI = 4', 6-diamidino-2-phenylindole; Ethidium Bromide = 2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide; FITC = Fluorescein isothiocyanate, Hoechst 33258 = 2'-[4-hydroxylphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole; Primulin = Direct yellow 59; Proflavin = 3,6-diaminoacridine.

Fluorochromes such as Acridine Orange (Hobbie et al., 1977), DAPI (Porter & Feig, 1980), Ethidium Bromide (Roser, 1980) and Hoechst 33258 (Paul, 1982) have been routinely used for the enumeration and characterization of microflora in environmental samples (Bitton et al., 1993; Bitton & Harvey, 1992; Kepner & Pratt, 1994). Harvey and colleagues have reported the use of DAPI as a tagging agent in a series of field and laboratory studies on a sandy, freshwater aquifer on Cape Cod, MA. These studies were aimed at elucidating the transport characteristics of bacteria and like-sized particles (Bales et al., 1995; Harvey et al., 1989; Harvey et al., 1995), determining the role of various environmental and physicochemical variables (Scholl & Harvey, 1992; Harvey et al., 1993), and modeling bacterial transport behavior in porous media (Harvey & Garabedian, 1991). However, little attention has been given to the potential effects of staining on the transport characteristics of bacteria.

Microbial transport through porous media is modulated by surface-to-surface interactions between microorganisms and solid matrices (Camper et al., 1993; Gannon et al., 1991; Schneider et al., 1994; Scholl et al., 1990; Wan et al., 1994). Under controlled conditions, surface properties such as electrostatic potential (surface charge) and hydrophobicity play a predominant role in determining microbial transport characteristics (Loosdrecht et al., 1990b). Potentially, staining may alter the transport characteristics of bacteria by inducing changes to their surface properties. Therefore, the accuracy and reliability of bacterial transport data could be seriously compromised if the effect of staining is not characterized and understood. Ideally, stains that do not affect the surface properties or transport of bacteria under consideration should be used.

CHAPTER 3
EFFECT OF FLUOROCHROMES ON BACTERIAL SURFACE PROPERTIES

Introduction

Transport of microorganisms in porous media such as soil, aquifers, and filtration systems is important in bioremediation of contaminated soils and groundwater, monitoring and containment of pathogens in the subsurface environment, and water and wastewater treatment by granular media filters (Bales et al., 1995; Bitton & Harvey, 1992; Duncan, 1988; Pell & Nyberg, 1989). Ideally, the microorganisms of interest should be used in microbial transport studies. This requires some means of tagging the test microorganisms so they can be distinguished from their indigenous counterparts in natural or engineered systems where microbial populations are present.

Potential methods of tagging microorganisms include radioactive labels (Hilbert & Logan, 1995), molecular or antibiotic markers (Ijzerman et al., 1993; Manz et al., 1993), and fluorochromes (Bales et al., 1995; Harvey et al., 1989). The former three methods have several disadvantages which limit their applicability in transport studies. Radioactive labeling methods are undesirable due to cost and environmental and safety concerns. Molecular labels such as fluorescent antibodies and PCR-markers have limited availability and their use is technically demanding. Use of unique plasmids or reporter genes is also technically demanding, and in addition, may require containment. Limitations of antibiotic-resistance as means of tagging bacteria include possible loss or transfer of resistance-encoding genes to indigenous bacteria and the ecological consequences of releasing antibiotic resistance genes to the environment (Coghlan, 1993; Davies, 1994).

Fluorochromes such as Acridine Orange (Hobbie et al., 1977), 4', 6-diamidino-2-phenyline-indole (DAPI) (Porter & Feig, 1980), ethidium bromide (Roser, 1980), Hoechst 33258 (Paul, 1982), and hydroethidine (Harvey et al., 1995) are traditionally used for the enumeration and characterization of microflora in environmental samples. They may also be used as a simple, direct and safe alternative for bacterial tagging, especially in microbial transport studies of short-term nature, such as in the testing, optimization and modeling of filtration systems for water and wastewater treatment. For example, DAPI has recently been used as a tagging agent in microbial transport studies by Harvey and colleagues (Bales et al., 1995; Harvey et al., 1989; Harvey & Garabedian, 1991; Harvey et al., 1993; Scholl & Harvey, 1992). Fluorochromes are attractive not only because they are readily available and simple to use, but also because they enable direct visual counting of tagged cells, which can mean shorter time and greater accuracy when used properly.

So far, no attention has been given to the effects of the staining procedure on the transport characteristics of microorganisms. Microbial transport through porous media is modulated by surface-to-surface interactions between microorganisms and solid matrices (Camper et al., 1993; Gannon et al., 1991; Reynolds et al., 1989; Schneider et al., 1994). Properties such as surface charge and hydrophobicity play a predominant role in determining microbial transport characteristics (van Loosdrecht et al., 1990). Staining may change the surface properties of microorganisms and, possibly, their transport characteristics. Therefore, the accuracy and reliability of bacterial transport data could be seriously compromised if the effect of staining is not characterized and understood.

Ideally, stains that do not affect the surface properties or transport of bacteria under consideration should be used. Another important consideration is the stability of fluorescence in stained microbial cells, since duration of transport experiments (including necessary storage time) is limited by stability of the visual tag (fluorescence).

The present study was carried out to evaluate the effect of selected fluorochromes on bacterial surface characteristics and interaction with granular media. Stability of fluorescence of stained cells was also examined. Surface properties were characterized by zeta potential and microbial adhesion to hydrocarbons. The effect of staining on interactions between bacteria and porous media was evaluated in terms of removal of bacteria in batch adsorption tests using sand coated with $Al(OH)_3$ to enhance adsorption. Staining effects on pure cultures and on natural wastewater bacteria were compared in batch adsorption tests. Our results indicate that staining can change the surface properties of bacteria and also affect their removal by surface-modified sand, and that the magnitude of these effects is stain-specific. The stained bacteria were stable for relatively short periods of time (hours to days), hence stability considerations may limit the use of stained cells in extended transport experiments.

Materials and Methods

Fluorochromes

Characteristics of the fluorochromes used in the present study are given in Table 3-1. Stock solutions of the fluorochromes were made in MilliQ water at concentrations given in Table 3-1, filter-sterilized (0.2 μm pore size), and stored according to manufacturers' recommendations.

Table 3-1. Fluorochromes used in this study

Fluorochrome*	Excitation light	Fluorescing color	Stock conc., mg mL^{-1}	Final conc., µg mL^{-1}
Acridine orange	Blue	Green, orange	5.0	500
DAPI	UV	Greenish blue	0.25	25
Ethidium bromide	Blue	Reddish orange	5.0	500
FITC	Blue	Bluish green	2.5	250
Hoechst 33258	UV	Bright blue	5.0	100
Primulin	UV	Greenish blue	1.0	100
Proflavin	Blue	Bright green	2.5	250

* Acridine Orange = 3, 6-bis[dimethylamino]acridinium chloride; DAPI = 4', 6-diamidino-2-phenylindole; ethidium bromide = 2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide; FITC = fluorescein isothiocyanate, Hoechst 33258 = 2`-[4-hydroxylphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole; primulin = Direct Yellow 59; Proflavin = 3,6-diaminoacridine.

Bacterial Strains and Growth Conditions

Model bacteria used in this study were *Escherichia coli* C3000 (ATCC 15597), an indicator of fecal pollution, and a hydrophobic strain of *Acinetobacter* (ATCC 31012) (Rosenberg, 1981). Batch cultures of *E. coli* C3000 and *Acinetobacter sp.* inoculated from frozen stock were grown overnight to early stationary phase. The 100-mL pure cultures were grown in 250-mL Erlenmeyer flasks with 120 rev min^{-1} shaking in Nutrient Broth (Sigma) at a pH of 7.2 and temperature of 35°C for *E. coli* and 30°C for *Acinetobacter sp.* Selected tests were also carried out using a natural mixture of wastewater bacteria that was obtained from a fresh sample of raw wastewater from the influent to the University of Florida Water Reclamation Facility. The wastewater was settled for 1 hr in a glass beaker covered with aluminum foil, and the supernatant was then transferred to a sterile plastic container for immediate use.

Preparation of Test Cells

Pure culture cells were harvested by centrifugation at 3000 x g for 10 min at 4°C, washed once and resuspended in filter-sterilized 10 mM phosphate buffer (van der Mei et al., 1993) to a concentration of approximately 2.5×10^8 cells mL^{-1}. The buffer pH was adjusted to 7.0 unless otherwise indicated. Wastewater bacteria were stained directly in the fresh wastewater supernatant. Bacterial suspensions were sonicated in a water bath sonicator (117W, Branson) for 3-5 minutes at room temperature to break up flocs, then distributed to sterile plastic vials. Aliquots of the fluorochrome stock solutions were added to the vials to give the final concentrations shown in Table 3-1. After a 30-min contact time, cells were washed twice in 2× volume of buffer with centrifugation at 7000 x g for 10 min at 4°C, and resuspended in buffer to desired concentrations. The stained cells were stored at 4°C for 24 hours before use, unless otherwise specified. Non-stained (control) cells were subjected to the same procedure.

Surface Modification of Sand

The fraction of 20 × 30 mesh Ottawa sand (Fisher) passing a U.S. Standard No. 25 sieve was collected to give sand particles in a size range of approximately 600-700 µm. The sand was coated with aluminum hydroxide to enhance adsorption of bacteria (Lukasik et al., 1996) according to the following procedure. Graded sand was rinsed thoroughly with deionized water, air dried, and then spread out shallowly in a plastic tray. A sufficient volume of 1.0 molal $AlCl_3\cdot6H_2O$ (Fisher) solution was added to just cover the sand. After

30 min., excess liquid was drained and the sand was air dried for 24 hr. The dry sand was then slowly transferred into a glass beaker containing 2 to 3 volumes of 3.0 N ammonium hydroxide. After 10 min of contact, excess liquid was decanted and the sand air dried. The dry sand was rinsed with deionized water until the supernatant was clear, air dried again, and stored at room temperature in sealed plastic bottles.

Batch Adsorption Tests

The batch adsorption tests were carried out at room temperature in 50-mL plastic centrifuge tubes affixed to a 70 cm diameter wheel that was rotated vertically at 30 rev min^{-1}. Initially, 5.0 grams of sand, followed by 24 mL of pre-stained or unstained (control) bacterial suspension ($\sim 5\times10^5$ cells mL^{-1}), were added to each tube. A 4.0-mL supernatant sample was withdrawn from each tube, then the tubes were rotated for 6 hr. Two sets of blanks, consisting of tubes with sand and buffer, but without bacteria, and tubes with buffer and unstained bacteria, but without sand, were run along with the other tubes. After mixing, the suspensions were settled for 10 min at room temperature, then 4.0 mL supernatant was collected from each tube. Samples were stored at 4°C for 24 hr before counting. The pre-stained samples were re-stained with the respective fluorochromes, and the controls and blanks were post-stained with DAPI, immediately before filtration and counting. All treatments were run in triplicate.

Epifluorescence Microscopy

Epifluorescence direct counts were carried out according to standard procedures (APHA et al., 1992; Sherr et al., 1993) with a Nikon Optiphot microscope equipped with appropriate filter blocks for desired light excitation and emission (see Table 3-1). Samples were sonicated for 3-5 min at room temperature immediately before filtration. One mL of sample was filtered through a sterile 0.20 µm pore size, black, nucleation track polycarbonate filter (Nuclepore, Costar Scientific Corp.), with a 0.45 µm pore size membrane filter (GN-6, Gelman) placed underneath to evenly distribute the vacuum. Filters were pre-wetted with filter-sterilized MilliQ water and a vacuum of 600 mm Hg applied. Three mL of buffer (pH 7.0) was added to the filter funnel immediately before applying vacuum to help disperse bacteria on the filter. Ten microscopic fields were counted per filter, and three filters were counted per sample. Sample concentrations typically gave 25-50 cells per microscopic field, hence the total number of cells counted per sample exceeded the minimum range recommended by Kepner & Pratt (1994).

Measurement of Zeta Potential

Zeta potential of stained and nonstained bacteria was measured at selected pH values using a model 501 Laser Zee meter (PEN KEM) according to the manufacturer's recommendations. This instrument derives zeta potential readings from electrophoretic mobilities, which are computed based on particle velocities in an applied electrical field. The particle velocities are detected using scattering of incident laser light by the particles. Immediately before each measurement, 4.0 mL of cell suspension was added to a plastic vial containing 100 mL of 10 mM phosphate buffer at a given pH of 2.0, 3.0, 4.0, 7.0, or 10.0. The vial was capped and shaken, then the pH was checked. Final cell concentration was approximately 10^7 cells mL^{-1}. Six readings were taken and averaged for each measurement. This procedure was repeated in triplicate for each treatment.

Microbial Adhesion to Hydrocarbons (MATH)

Kinetic MATH (Lichtenberg et al., 1985) was employed to measure bacterial surface hydrophobicity using a procedure modified from van der Mei et al. (1993). Stained and unstained cells were suspended in aliquots of 10 mM phosphate buffer previously adjusted to pH values of 2.0, 3.2, 4.0, 7.0, or 10.0. The pH was checked after cell addition. Final cell concentrations were approximately 10^8 cells mL^{-1}. Aliquots (4.0 mL) of the suspensions were distributed among sterile 13 mm × 100 mm glass test tubes, followed by the addition of 140 μL of hexadecane (Sigma) per tube to give a final concentration of 35 μL hexadecane mL^{-1}. The cell-coated oil droplets created by mixing the two phases float to the top when the two phases are allowed to separate, effecting partition of bacterial cells from the bulk (aqueous) phase to the oil/water interface (upper phase). Bacterial concentration in the aqueous phase was monitored by measuring the A_{550} of the aqueous phase in a spectrophotometer (Spectronic 21, Milton Roy). Initial absorbance (A_0) was measured before mixing. Subsequently, the aqueous and hydrocarbon phases were mixed by 10 seconds of vortexing, then allowed to separate for 10 minutes at room temperature, at which time the absorbance of the aqueous phase (A_t) was measured again. This procedure was repeated until a cumulative vortexing time of at least 60 sec was reached. The base-10 logarithm of the quantity ($A_t/A_0 \times 100$) was plotted against the cumulative vortexing time. From this plot, the initial removal rate, R_0, was derived by linear least-squares fitting to the initial, linear portion of the curve. R_0 was used as a measure of hydrophobicity.

Results and Discussion

Visualization and Stability

The four nucleic acid fluorochromes tested (Acridine Orange, DAPI, ethidium bromide, Hoechst 33258) gave satisfactory visualization of *E. coli* in terms of resolution and intensity, whereas the three cytoplasmic fluorochromes tested (FITC, Primulin, Proflavin) gave inferior visualization and were not considered further. Cells stained with DAPI showed an intense, stable, and uniform greenish blue color under epifluorescent illumination. There was little background in DAPI-stained samples, whereas noticeable background was encountered in samples stained with AO. Lack of specificity of AO has been noted by others (Ghiorse & Balkwill, 1983; Porter & Feig, 1980).

Counts of *E. coli* cells pre-stained with DAPI, Acridine Orange or Hoechst 33258 remained above 90% of the initial counts after one day of storage at 4°C (Fig. 3-1a). After two days of storage, only cells pre-stained with DAPI gave counts above 90% of initial counts. Counts of post-stained cells (controls), which indicate the total number of cells remaining upon storage, were not significantly different ($P<0.05$, two-tailed *t* test) from the DAPI-stained samples after two days. Similarly, Yu et al. (1995) reported that prepared slides of DAPI-stained samples could be stored at 4°C for 2 days with only a small sacrifice of the total cell numbers. Counts of all pre-stained cell samples fell well below 90% of the initial counts after two additional days. Counts of the post-stained controls fell slightly below 90% of initial counts after four days, which is not unexpected since decay can change cell numbers upon storage (Yu et al., 1995).

Similar trends were found with *E. coli* cells pre-stained with AO and DAPI when stored at 20°C (Fig. 3-1b). Counts of both AO- and DAPI-stained cells were close to 100% of initial counts after one day of storage at 20°C. No significant difference ($P<0.05$, two-tailed *t* test) was detected among AO- and DAPI-stained samples and control (non-stained cells also stored at 20°C but freshly-stained with DAPI for each counting) at this point. After two days of storage at 20°C, counts of AO-stained cells (below 85% of initial counts) were significantly lower ($P<0.05$, two-tailed *t* test) than that of the control (above 95% of initial counts). In contrast, counts of DAPI-stained cells (above 95% of initial counts) were statistically identical to that of the control. Counts of both AO- and DAPI-stained cells fell below that of the control after four and six days of storage at 20°C, although AO-stained cells suffered a much sharper decrease in numbers. Counts of DAPI-stained cells were higher when stored at 20°C than when stored at 4°C. This was probably due to different physiological states of test cells used in the two experiments.

Successful use of fluorochrome-based tagging methods requires fluorescence stability of pre-stained cells during the study period, including necessary storage time. Samples pre-stained with DAPI retained satisfactory fluorescence at 4°C or 20°C for up to 2 days without appreciable decrease in visual counts. Considering that most transport studies are conducted at temperatures in the proximity of 20°C, DAPI can be used to tag test bacteria, provided that the duration of transport study, including storage time, is no more than two days. Usage of samples pre-stained with any of the other nucleic acid fluorochromes tested should be limited to one day.

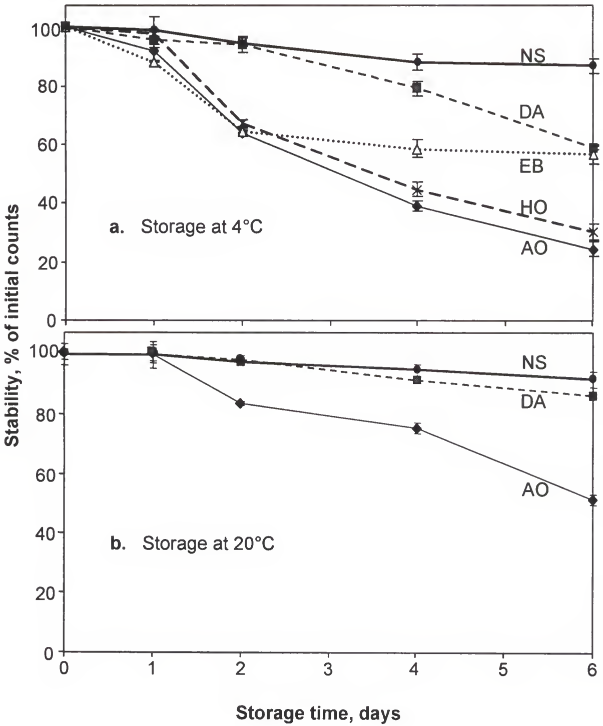

Fig. 3-1. Effect of storage time on counts of bacteria stained with nucleic acid fluorochromes (error bars represent ± 1.0 S.D.; NS = nonstained, DA = DAPI, EB = ethidium bromide, HO = Hoechst 33258, AO = Acridine Orange)

Removal of Bacteria in Batch Adsorption Tests

Preliminary batch adsorption tests with *E. coli* indicated that a steady-state was approached after two hours of mixing, and that adsorption remained essentially unchanged between two and eight hours of mixing (data not shown). Accordingly, we used 6 hr of mixing in subsequent experiments to ensure that a steady-state was reached. Others have reported equilibrium times of 1-3 hr (Mills et al., 1994). The sand quantities used in the tests were selected to give removal percentages in the vicinity of 50% when initial bacterial concentrations were on the order of 10^5 cells mL^{-1}.

As shown in Figure 3-2a, pre-staining *E. coli* cells with DAPI had negligible effect on removal relative to non-stained cells (control). Acridine Orange and Hoechst 33258 increased removal significantly relative to non-stained cells ($P<0.05$, two-tailed t test). Ethidium bromide decreased removal, although to a statistically insignificant degree. Use of artificial groundwater (McCaulou et al., 1994) as the liquid medium gave similar results (Fig. 3-2d). Pre-staining with DAPI also had a negligible effect on removal of *Acinetobacter sp.* relative to non-stained cells (Fig. 3-2b). Pre-staining with each of the other fluorochromes significantly increased removal. Similarly, in the case of wastewater bacteria, pre-staining with DAPI did not affect removal, while AO had a significant effect ($P<0.05$, two-tailed t test) on removal, relative to non-stained cells (Fig. 3-2c). Most of the bacteria in the wastewater sample were coccoid and rod-shaped. Only a few filamentous bacteria were found. Batch removal test results were thus consistent for both pure bacterial cultures and wastewater bacteria.

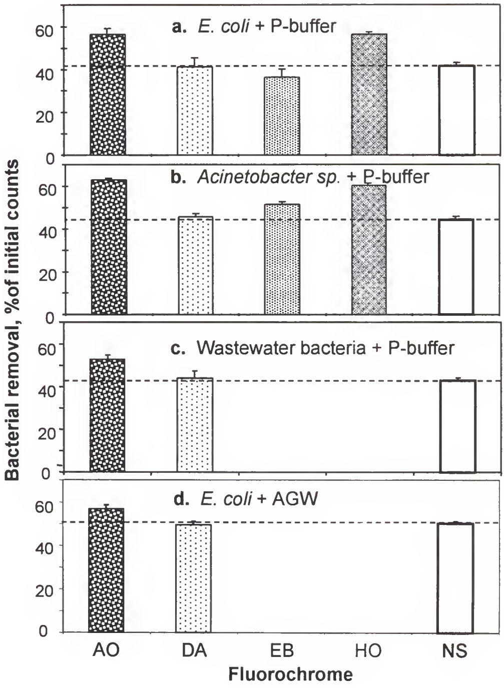

Fig. 3-2. Effect of staining on removal of bacteria in batch adsorption tests (error bars represent ± 1.0 S.D.; NS = nonstained, DA = DAPI, EB = ethidium bromide, HO = Hoechst 33258, AO = Acridine Orange; P-buffer = 10 mM potassium phosphate buffer, AGW = artificial groundwater)

Based on these results, we selected DAPI for further testing with regard to its effect on bacterial surface properties. Acridine Orange was also tested for comparison because it is widely used in direct-counting procedures (Kepner & Pratt, 1994).

Effect of Staining on Zeta Potential (ζ)

The ζ potential of *E. coli* and *Acinetobacter sp.* cells decreased as pH was varied over the range of pH 2 to 10, with isoelectric points (IEPs) of pH 3.1 and pH 3.3, respectively (Fig. 3-3). These are within the range of pH values expected for most bacteria (Harden & Harris, 1953). The effect of pre-staining on ζ potential over the pH range investigated was evaluated using paired *t*-tests. AO significantly increased ζ potential of *E. coli* cells, while significantly decreasing ζ potential of *Acinetobacter sp.* ($P<0.05$), except at pH 3.0 (where no significant effect was observed on either bacterium). In contrast, DAPI did not significantly affect the ζ potential of either bacterium, except at pH 10.0 (where a significant effect was observed on *E. coli*). Based on DLVO theory (Adamson, 1990), the direction of the change in ζ potential caused by AO is not completely consistent with the adsorption test results. AO increased ζ potential of *E. coli* and decreased ζ potential of *Acinetobacter sp.*, but enhanced removals of both bacterial species. This may indicate that electrostatic interactions are not dominant in this system.

Effect of Staining on Surface Hydrophobicity

The microbial adhesion to hydrocarbons (MATH) test is based on the partitioning of bacteria between aqueous and hydrocarbon phases (Rosenberg et al., 1980). Because of difficulties in obtaining equilibrium, a kinetic approach is considered more quantitative

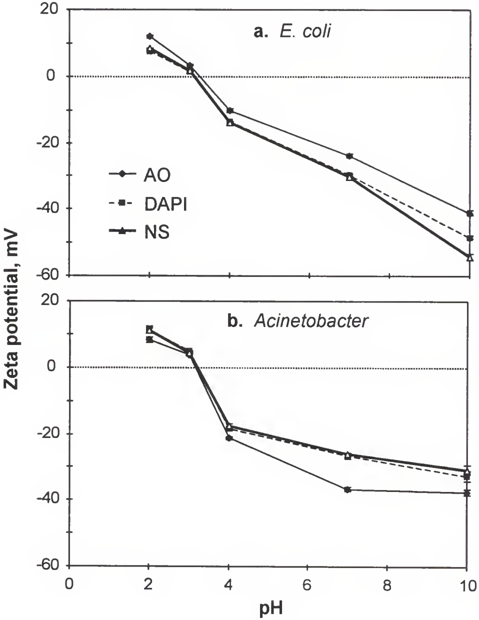

Fig. 3-3. Effect of staining on zeta potential of bacterial cells (error bars represent ± 1.0 S.D.; NS = nonstained, DA = DAPI, AO = Acridine Orange)

(Lichtenberg et al., 1985). In the present study, cell surface hydrophobicity was measured in terms of R_0 from the kinetic MATH test (van der Mei et al., 1993). R_0 varied with pH, with the greatest R_0 for *E. coli* occurring at pH 3.2 (Fig. 3-4a) and the greatest R_0 for *Acinetobacter sp.* occurring at pH 4.0 (Fig. 3-4b). AO noticeably decreased the R_0 of both *E. coli* and *Acinetobacter sp.* throughout the pH range investigated (pH 2-10). In contrast, DAPI caused a noticeable decrease only at the pH where maximum R_0 values of the nonstained cells were observed. At these values (pH 3.2 for *E. coli* and pH 4.0 for *Acinetobacter sp.*), the effect of staining on R_0 was significant ($P<0.05$, two-tailed *t* test) with either fluorochrome.

Since bacterial adhesion is primarily influenced by the interplay of electrostatic and hydrophobic interactions (van Loosdrecht et al., 1990), the most sensitive measurement of bacterial surface hydrophobicity (in terms of R_0) should be at a pH where electrostatic interactions are minimized (i.e., either the bacteria or the hydrocarbon is uncharged). Hexadecane is negatively charged in aqueous suspensions (including potassium phosphate solutions) and its zeta potential has been shown to be also pH-dependent, with an IEP at pH 3.2 (Geertsema-Doornbusch et al., 1993; Medrzycka, 1991; van der Mei et al., 1993). Absence of charge effect would allow hydrophobic interactions to dominate over electrostatic interactions. Observation of the maximum R_0 for *E. coli* at its measured IEP (pH 3.1) supports this hypothesis. Similar pH-dependence of R_0 has been reported for other bacterial strains (van der Mei et al., 1993). Occurrence of the maximum R_0 for *Acinetobacter sp.* at pH 4.0, rather than at the IEP (pH 3.3) measured in this work, was surprising. This might have resulted from subtle physiological differences between the batch cultures used for the two different tests. Both DAPI and AO significantly decreased

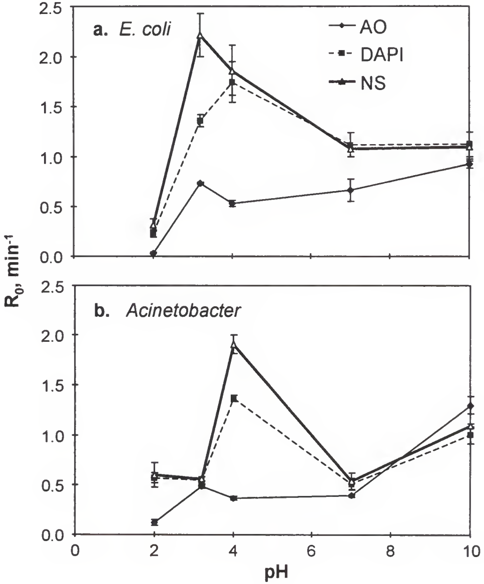

Fig. 3-4. Effect of staining on hydrophobicity of bacterial cells (error bars represent ± 1.0 S.D.; NS = nonstained, DA = DAPI, AO = Acridine Orange)

cell hydrophobicity, although the latter showed the greater effect. Decrease in hydrophobicity of bacterial cells could lead to stronger interaction with the more hydrophilic, charged surface of the $Al(OH)_3$-coated sand, and therefore may at least partially account for increased adsorption.

Conclusion

This study has shown that nucleic acid fluorochromes can affect the surface properties of stained bacterial cells and alter interaction of the cells with surfaces. This finding is of importance in microbial transport studies using fluorescence-tagged bacteria, since transport through porous media is modulated by surface properties of the microorganisms as well as the solid matrices (Gannon et al., 1991; Scholl et al., 1990). Another relevant finding is the time-dependence of fluorescence of stained cells that limits the use of tagged cells in long-term transport studies. Of the four nucleic acid fluorochromes tested, DAPI had minimal effect on surface properties and no significant impact on adsorption of bacteria to aluminum hydroxide-coated sand. The minimal effect of DAPI is expected given its DNA-specificity (Coleman, 1980; Porter & Feig, 1980). In contrast, AO, which is considered to be a nonspecific fluorochrome capable of binding to cell wall components (cytoplasm, flagella) as well as DNA and RNA (Ghiorse & Balkwill, 1983; Paul & Myers, 1982; Sieracki & Viles, 1992), strongly affected bacterial surface properties and adsorption to surfaces. These trends were revealed by batch adsorption tests using either pure cultures (*E. coli*, *Acinetobacter* sp.) or wastewater bacteria.

CHAPTER 4
LONG-TERM EVALUATION OF ALUMINUM HYDROXIDE-COATED SAND UNDER WASTEWATER CONDITIONS

Introduction

Removal of fine particles such as bacteria and viruses by filtration through porous media is difficult, since these biocolloids carry a negative surface charge in the pH range of natural waters (Loder and Liss, 1985; Marshall, 1976). Most filter media (e.g., sand, diatomaceous earth) are also negatively charged in this pH range. Coating of filter media with metallic hydroxides, oxides, or peroxides enhances removal of bacteria, viruses and turbidity from water and wastewater (Ahammed and Chaudhuri, 1996; Farrah et al., 1991; Gerba et al., 1988; Mills et al., 1994). The primary function of the coatings is to make the surface of the filter media more electropositive, thus facilitating colloidal attachment.

When coated filter media are placed in service, the metallic hydroxide surface will be subject to dissolution, attrition, change in surface area and roughness, and chemical or biological fouling, all of which can lead to aging (decrease in removal capacity) of the coated media. Thus far, effects of these aging phenomena on media performance have been largely overlooked. Ahammed and Chaudhuri (1996) reported that coatings of iron hydroxide or iron and aluminum hydroxide on sand continued to effectively remove fecal coliforms from water after 50 days of intermittent operation. The effect of continuous exposure of coated media to water was not investigated, however. In water and wastewater treatment systems containing organic substrates, a large potential exists for biogrowth on filter media. Biogrowth in slow sand filters is essential for effective removal of microorganisms (Schuler et al., 1991). Development of biofilm on glass, polycarbonate, and granular activated carbon surfaces enhanced removal of bacteria (Banks & Bryers, 1992) and bacterium-sized particles (Drury et al., 1993; Rittmann & Wirtel, 1991; Sprouse & Rittmann, 1990). The effect of biogrowth on the performance of surface-modified filter media has not been reported.

This study is the first to examine the effect of long-term exposure to wastewater on the effectiveness of metallic hydroxide coating on filter media. The coating studied was aluminum hydroxide on sand. Wastewater was treated to allow biogrowth in one set of columns and to prevent biogrowth in a parallel set of columns. Performance of the coating was measured in terms of *Escherichia coli* removal. The results indicated a progressive decline of removal capacity of the coated sand in response to continuous exposure to wastewater flow. The removal capacity of the coated sand decreased to the level of the uncoated sand over a period of 118 days. Biogrowth on the filter media exacerbated this decline. These results provide important information on the stability and effective lifetime of the surface coating under wastewater conditions. This information is required to assess the technological potential, as well as economical implications, of metallic hydroxide coating of filter media.

Materials and Methods

Surface Modification of Sand

The fraction of 20 × 30 mesh Ottawa sand (Fisher Scientific, Springfield, NJ) passing a U.S. Standard No. 25 sieve was collected to obtain sand particles in the size range of approximately 600-700 µm diameter. The graded sand was coated with aluminum hydroxide to enhance adsorption of bacteria (Lukasik et al., 1996) according to the following procedure. A 1,000 g batch of sand was rinsed with deionized water until the supernatant became clear, air dried, then spread out to form a 3-cm thick layer in a plastic tray. A volume of 1,000 mL of 1.0 molal $AlCl_3{\cdot}6H_2O$ (Fisher Scientific) solution was added to the tray. After 30 min of contact, the excess solution was drained and the sand was air dried in the plastic tray for 24 hr. The dried sand was transferred slowly into a 2.0-L glass beaker containing 1,000 mL of 3.0 N ammonium hydroxide and contacted for 10 min. The excess liquid was drained, then the sand was air dried in the plastic tray. The dried sand was rinsed vigorously with deionized water to remove loose precipitate, then air dried again in the plastic tray. Batches of coated sand were combined, mixed thoroughly, then stored at room temperature in sealed plastic bottles until use. A portion of the coated sand was not exposed to wastewater. Aliquots of this sand, which we term "positive control," were tested in parallel with all samples. Similarly, uncoated sand that was not exposed to wastewater was used as the "negative control."

Exposure of Al-coated Sand to Wastewater in Continuous Flow Columns

Aluminum hydroxide-coated sand was exposed to wastewater in continuous flow-through columns that were operated in an upflow mode for 118 days. The wastewater used in this study was the biologically treated tertiary effluent (chlorinated upstream to the chlorine contact basin) of the University of Florida Water Reclamation Facility. The typical wastewater composition before chlorination was: pH, 7.2; alkalinity, 44 mg/L as $CaCO_3$; turbidity, 0.7 NTU; NO_3-N, 1.2 mg/L; NH_3-N, 0.2 mg/L; total Kjeldahl nitrogen, 1.0 mg/L; orthophosphate-P, 1.2 mg/L; total phosphate-P, 1.8 mg/L; Ca^{2+}, 73 mg/L; Mg^{2+}, 8 mg/L; conductivity, 795 Tmhos/cm. Sand was packed into the acrylic columns (3.2 cm I.D. × 1.5 m) to an initial depth of 0.75 m. A total of 6 columns were used in the experiment, three exposed to chlorinated wastewater to inhibit biogrowth and the other three to dechlorinated wastewater to permit biogrowth (Fig. 4-1). This was designed to assess the effect of biogrowth on the surface properties and removal capacity of the surface-modified sand. Fresh chlorinated feed was obtained daily by dosing wastewater with 10 mg L^{-1} ammonia nitrogen, followed by titration with fresh sodium hypochlorite (Clorox Professional Products, Oakland, CA), to give a combined residual chlorine concentration of 2 mg L^{-1}. A combined residual was used to avoid rapid dissipation of the chlorine during storage of wastewater in the feed tank. Dechlorinated wastewater was obtained by dosing treated wastewater with sodium thiosulfate before it was pumped into the columns. The upflow rate in the columns was 57 mL min^{-1}, which gave a superficial velocity (flow rate divided by the cross section area of the column) of 1.2 mm s^{-1}. The pH and temperature of the feedstocks were in the ranges of 6.6-7.0 and 22-25°C, respectively. Prior to the long-term experiment, a preliminary 45-day trial was carried out utilizing four small acrylic columns (1.5 cm I.D. × 1.0 m). The columns were packed to an initial depth of 0.5 m with $Al(OH)_3$-coated sand. Only dechlorinated wastewater was fed to the columns. The flow rate in each column was 20 mL min^{-1}, which gave a superficial velocity of 1.9 mm s^{-1}.

Rubber stoppers with tubing connectors were used to seal both ends of the columns. A fine nylon screen was used at the bottom of each column to prevent sand from entering the tubing. Columns and tubing were covered with black plastic to prevent growth of

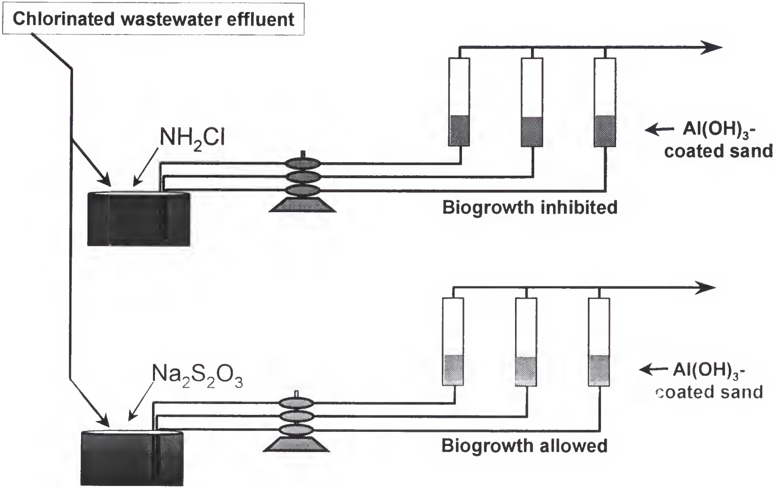

Fig. 4-1. Schematic diagram of column setup for exposing surface-modified sand to dechlorinated or chlorinated wastewater

algae. The columns were backwashed for 15 min every 72 hr at a superficial velocity of 17 mm s^{-1}, which was sufficient to completely fluidize the sand. Before each sampling, the columns were backwashed and then drained. Sand was emptied from each set of columns, combined, mixed, and sampled. The remaining sands were returned to the respective columns and feeding was resumed. Samples were rinsed with dechlorinated wastewater (filter-sterilized) to remove loosely attached biomass, then divided into subsamples. The subsamples were kept at 4°C for up to 24 h prior to analysis.

Preparation of Test Bacteria

Batch cultures of *Escherichia coli* C3000 (ATCC 15597) were grown overnight to early stationary phase in Nutrient Broth (Sigma Chemical, St. Louis, MO) at 35°C with shaking at 120 rev min^{-1}. A bacterial suspension of 50 mL from each batch was centrifuged at 3000 × g for 10 min at 4°C to harvest the cells. The cells were resuspended in filter-sterilized MilliQ water to $A_{550} = 0.40$ and stained for 30 min with 4', 6-diamidino-2-phenyline-indole (DAPI) (Porter & Feig, 1980) at a final concentration of 25 µg/mL. Subsequently, the stained cells were harvested, washed in 2 × volume (100 mL) of artificial ground water (AGW) (McCaulou et al., 1994) with vortexing, and re-harvested by centrifugation at 7000 × g for 10 min at 4°C. The cells were resuspended in artificial groundwater (AGW) (pH 7.0) and kept at 4°C until use.

Batch and Column Removal Tests

Batch removal tests were carried out using samples of $Al(OH)_3$-coated sand that had been exposed to wastewater in the columns. Parallel tests were also run with $Al(OH)_3$-coated (positive control) and uncoated (negative control) sands that were not exposed to wastewater. Initial bacterial concentrations were adjusted to 10^5-10^6 cells mL^{-1}. The test bacteria were contacted for 2 hr with the respective sands in 50-mL plastic centrifuge tubes affixed to a 70 cm diameter wheel that was rotated vertically at 30 rev min^{-1} at room temperature. Each test tube contained 5 g of wet sand (rinsed with AGW) and 20 mL of bacterial suspension. Blanks containing bacterial suspension but no sand were run along with the other tubes to obtain initial counts. After mixing, sand in the tubes was allowed to settle for 10 min at room temperature, then supernatants were sampled.

Parallel filtration tests were carried out on day 1 and 118 samples in four acrylic columns (1 m length × 1.5 cm I.D.). The columns were packed with sands from the same samples and controls used in the corresponding batch removal tests. Glass wool was used at either end of the columns to prevent loss of filter media. The porosity of the packed media was 40%. Columns were run in an upflow mode at a flow rate of 20 mL min^{-1}. The packed columns were initially rinsed with 20 pore volumes of AGW (pH 7.0). Influents to the four columns were drawn from a tank containing bacterial suspension adjusted to the same cell concentration (10^5-10^6 cells mL^{-1}) using bacteria from the same preparations used for the corresponding batch removal tests. The influent tank was continuously mixed with a paddle stirrer. Composite effluent samples were collected at the 71st through 75th pore volumes to obtain steady-state data (Kucukcolak et al., 1997). Composite influent samples were taken from the influent lines while pore volumes 71-75 were fed to the columns. The batch and column removal tests were run in triplicate. Samples were stored at 4°C for up to 24 hr until enumeration.

Bacterial Enumeration

Direct counts of bacterial samples were obtained within 48 hours (preliminary experiments showed that cell losses were less than 5% after this length of storage) using epifluorescence microscopy (APHA et al., 1992; Sherr et al., 1993). The samples were not re-stained, hence only pre-stained test cells were counted. This prevented interference from indigenous cells (background contamination or sloughed biomass). A Leitz Microlab microscope, equipped with appropriate filter blocks for desired light excitation and emission, was used for visualization and counting. Samples were vortexed vigorously to mix well immediately before filtration. A 1.0-mL aliquot of sample was filtered through a sterile 0.20 µm pore size, black, nucleation track, Nuclepore polycarbonate filter (Costar Scientific, Cambridge, MA), with a 0.45 µm pore size (GN-6) membrane filter (Gelman Sciences, Ann Arbor, MI) placed underneath to evenly distribute the vacuum. Filters were pre-wetted with filter-sterilized MilliQ water and a vacuum of 600 mm Hg applied. Three mL of filter-sterilized MilliQ water were added to the filter funnel immediately before applying vacuum to help disperse bacteria on the filter. Ten microscopic fields were counted per filter. Sample concentrations typically gave 25-50 cells per field, hence the total number of cells counted per sample exceeded the minimum range recommended by Kepner & Pratt (1994).

Protein Assay

The Lowry method (Lowry et al., 1951), as modified (Bensadoun and Weinstein, 1976; Peterson, 1977), was employed, based on the procedure described in the Protein Assay Kit from Sigma Chemical Company (Sigma Diagnostics / Procedure No. P 5656), to estimate protein accumulation due to biogrowth on sand exposed to wastewater. Twenty grams of wet sand were extracted in 8.0 mL of 0.1 N NaOH in sterile 50 mL plastic centrifuge tubes. The tubes were vortexed vigorously for 30 sec and let stand at room temperature for 5 min. These steps were repeated, then the tubes were placed in -40°C freezer for at least 30 min. After freezing, the tubes were thawed in a 50°C water bath for 10 min, followed by vortexing for 30 sec. The supernatants were transferred to 13 x 100 mm glass test tubes and allowed to settle for 30 min. The supernatants were then distributed in 2.0-mL aliquots to triplicate 15-mL plastic centrifuge tubes. Protein standards (bovine serum albumin from Sigma Chemical) were made in the concentration range of 25-400 μg mL^{-1}. Filter-sterilized MilliQ water (2.0 mL) was used in a separate centrifuge tube as a blank. Supernatant samples, standards and blank were run together through the same steps according to instructions provided in the Sigma kit. Absorbance was measured at 750 nm after allowing color to develop at room temperature for 30 min.

Scanning Electron Microscopy (SEM)

SEM was carried out in parallel with protein assay (i.e., at 1, 15, 62, 97, and 118 days) to visualize biogrowth. Sand samples were fixed in fresh 2.5% glutaraldehyde in phosphate buffered saline (PBS, pH 7.2) for 15-20 min at room temperature, washed in PBS, 2 times, 5 min each, and then subjected to post-fixation at room temperature in 4% osmium tetroxide (OSO_4) in PBS for 10 min or 4% OSO_4 in 0.1 M cacodylate buffer for 5 min. The fixed samples were rinsed twice with deionized water for 5 min each time, then dehydrated successively in a graded ethanol series (50%, 75%, 95%, 100%) for 5 min in each step, followed by two 5-min changes in hexamethyldisilizane (HMDS). The samples were subsequently air-dried on filter paper placed in petri dish, mounted on aluminum support using colloidal graphite with iso-propanol as adhesive, then oven-dried at 60°C, and finally sputter-coated with gold on an IB-2 ion coater (Eiko Engineering) according to manufacturer's recommendations. The gold-coated samples were viewed on a Hitachi S-4000 Field Emission SEM at 6 kV. Images were saved digitally using a Advanced Database Systems ImageSlave System. Final prints were printed on a Kodak 8600 PS Dye Sublimation printer.

Metal Content analysis

Sand samples (in triplicate) were digested in aqua regia (1:2:2::HCl:HNO_3:H_2O) for 20 min (10 g sand + 25 mL aqua regia). The mixture was heated to near the boiling point. The digestate was diluted to 1,000 mL, then filtered through a 0.2 μm pore size filter. The filtered digestate was then used for metal content analysis by ICP.

Granular Surface Potential

Surface charge is a key variable affecting bacterial interactions with granular media. Zeta potential, which approximates surface charge, was determined for each sample using a streaming potential apparatus that was constructed at the University of Florida. The apparatus consisted of a flow-through cell that was packed with the granular media to be analyzed and a tank to supply electrolyte (1.0×10^{-4} M KCl) to the flow-through cell. A water manometer was used to monitor the pressure drop across the cell and a Keithly 610c electrometer was used to measure the electrical potential between two silver chloride electrodes which preceded and followed the granular sample. The sample cell was made from a clear polycarbonate pipe with a ¾ inch inner diameter and a length of 20 cm (8 in.). The electrodes consisted of 99% pure 18 gauge silver wire and 40 mesh silver gauze (Newark Wire Cloth Co.) which was cut to match the 1.9 cm (¾ in.) circular diameter of the sample pipe. The wire and mesh were spot welded together so that the silver wire extended perpendicular from the center of the circular mesh piece. The mesh portion of the electrode was then anodized in 0.1 M HCl for 1 hour using a copper cathode and a 5 mA current. The finished electrode was then allowed to stabilize overnight in distilled water. Electrodes were regenerated every two to three weeks to ensure proper performance.

The sample cell was filled completely with sand. Uniform packing was obtained via tap and fill methodology. The sample cell was then flushed with carbon dioxide and then the KCl electrolyte solution to remove any potential air pockets. The actual physical operation of the streaming potential device allowed for a $1.0\text{x}10^{-4}$ M KCl salt solution to flow through the granular sample under investigation. Pressure was used as the controlled variable in these measurements and was allowed to range over the entire laminar flow regime. Electrodes at the edges of the sample monitored the voltage, ΔE, generated as the charged ions in the KCl solution pass the charged sample surface under investigation. This voltage varied as a function of pressure drop, ΔP. The slope of the measured voltage and pressure values was then used along with the measured solution conductivity, K, to calculate the corresponding zeta potential (mV), ς, with the Smoluchowski relation (Overbeek, 1952):

$$\zeta = \frac{4\pi \mu K}{\varepsilon \varepsilon_0} \frac{\Delta E}{\Delta P} \qquad (1)$$

where ε is the dielectric constant of the medium, ε_0 is the dielectric constant of a vacuum, and μ is the viscosity of water. Due to the weak electrolytic nature of the solution used in these measurements, the zeta potential is assumed to be very close to the surface potential and is the value used in the DLVO calculations.

The above mentioned apparatus typically provides a reproducibility within an error around 10% of the measured zeta potential. This error value was based upon the 95% confidence interval for the solution conductivity and the pressure-voltage slope variables used in the Smoluchowski equation. Standard deviations between samples were generally determined to be less than 3%.

Results and Discussion

Biogrowth Development

The protein content of $Al(OH)_3$-coated sand exposed to chlorinated wastewater remained at background levels throughout the 118 days of the experiment (Fig. 4-2). Similarly, no bacteria were found on this sand using SEM (data not shown). In contrast, both protein assay (Fig. 4-2) and SEM (Fig. 4-3) indicated time-dependent biogrowth on the sand that was exposed to dechlorinated wastewater. The protein content was significantly above background levels ($P < 0.05$, *t* test) after 15 days. It increased exponentially through day 97, after which the rate of increase declined (Fig. 4-2). This time-dependent biogrowth could be described by a logistic curve. The protein content after 118 days was about one-half of what we previously obtained for filter sand samples from the Kanapaha Water Reclamation Facility in Gainesville, Florida, which has for over

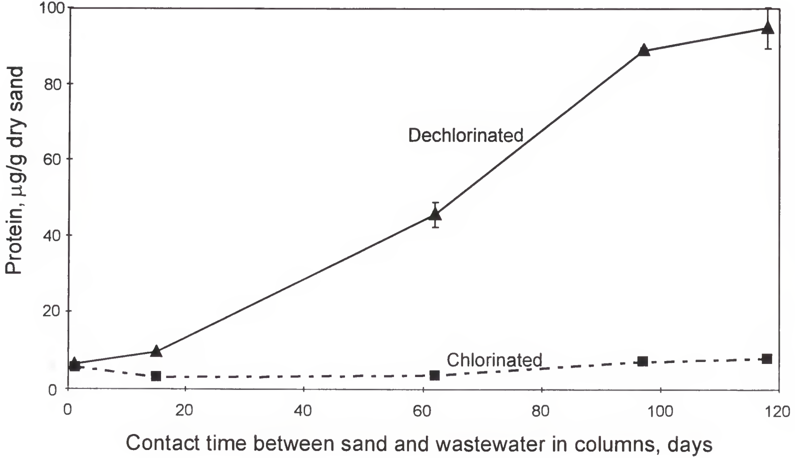

Fig. 4-2. Development of biogrowth on aluminum hydroxide-coated sand exposed to dechlorinated or chlorinated wastewater as measured by protein content. Error bars represent ±1.0 S.D.

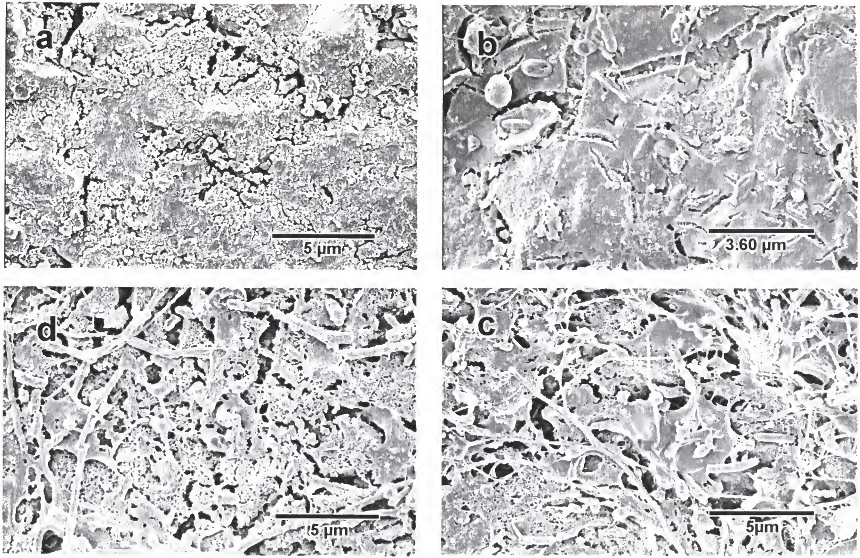

Fig. 4-3. Development of biogrowth on aluminum hydroxide-coated sand exposed to dechlorinated wastewater as observed by SEM - a. 1 day; b. 62 days; c. 97 days; d. 118 days.

25 years received clarified effluent from an activated sludge process without upstream chlorination.

SEM examination of sand exposed to dechlorinated wastewater indicated that bacteria on the sand surface were scarce initially, but increased in number with exposure time (Fig. 4-3). Surface coverage remained low even as protein content approached a stable level. Early biogrowth consisted of single, mostly rod-shaped bacterial cells (Fig. 4-3b). As the length of exposure (continuous passage of wastewater) increased, biogrowth composition became more diverse, with an increasing presence of filamentous microbes and polymeric network-like structures (Fig. 4-3c, 3d).

Metal Content and Zeta Potential of Test Sands

Coating the sand with $Al(OH)_3$ increased the Al content from approximately 0.05 to 0.4 mg Al/g dry sand. The Al content of the coated sands decreased by approximately one-fourth over the first two weeks of exposure to wastewater, then remained approximately constant but well above (approximately 6-fold) the level of uncoated sand (negative control) throughout the rest of the experiment (Fig. 4-4). This initial loss of coating may be attributed to a combination of attrition and leaching of loosely attached precipitates. Biogrowth does not appear to affect Al content, since there was no significant differences ($P < 0.05$, paired t test) in Al content between the sand exposed to dechlorinated wastewater vs. the sand exposed to chlorinated wastewater.

The zeta potential of $Al(OH)_3$-coated sand, before exposure to wastewater, was markedly higher than that of uncoated sand (above +20 mV vs. below –90 mV) (Fig. 4-5). Zeta potential of the coated sands decreased to the range of -50 to -65 mV after one day

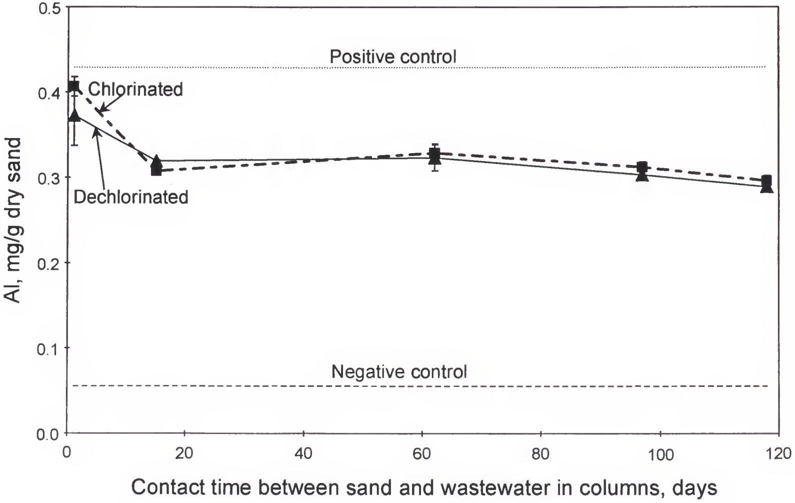

Fig. 4-4. Metal content of aluminum hydroxide-coated sand exposed to dechlorinated or chlorinated wastewater. Positive control = aluminum hydroxide-coated sand that was not exposed to wastewater; negative control = uncoated sand that was not exposed to wastewater. Error bars represent ±1.0 S.D.

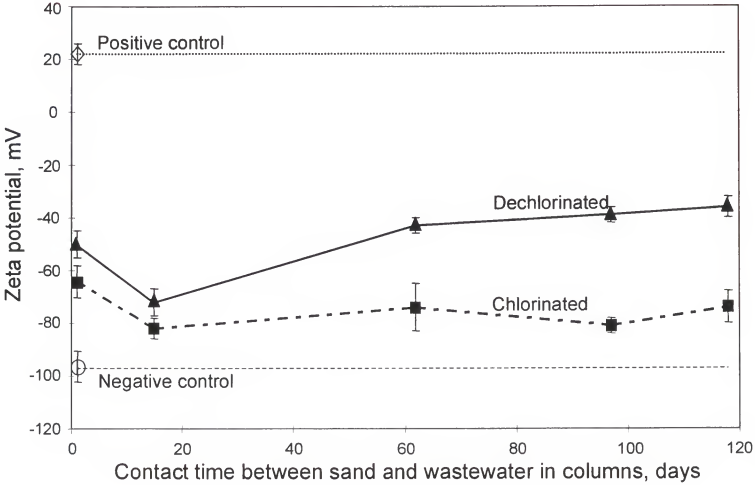

Fig. 4-5. Zeta potential of aluminum hydroxide-coated sand exposed to dechlorinated or chlorinated wastewater. Positive control = aluminum hydroxide-coated sand that was not exposed to wastewater; negative control = uncoated sand that was not exposed to wastewater. Error bars represent ±1.0 S.D.

of exposure to wastewater and further decreased to below -70 mV over the next two weeks. This was still significantly ($P < 0.05$) above the negative control. After this initial drop, zeta potential of coated sand exposed to chlorinated wastewater remained approximately constant, whereas zeta potential of coated sand exposed to dechlorinated wastewater gradually became less negative. The trend in zeta potential of coated sand exposed to chlorinated wastewater was similar to the trend in metal content. The increase in the positive nature of the zeta potential of coated sand exposed to dechlorinated wastewater paralleled the development of biogrowth. Previously, Rao et al. (1993) observed that the zeta potential of bacteria-coated apatite minerals was intermediate between that of the minerals alone and of the bacteria alone. We can thus attribute the increased zeta potential of sand with biogrowth to an "averaging" of the zeta potential of bacteria (typically from -10 to -60 mV) (Achouak et al., 1994; Nicholov et al., 1993; Vitaya & Toda, 1991) and that of the coated sand exposed to wastewater (around -80 mV, as seen in Fig. 4-5).

Removal of *E. coli*

Coated sand (positive control) was significantly better than uncoated sand (negative control) in the removal of *E. coli* (approximately 45-60% vs. 10-20%) (Fig. 4-6). Performance of coated sand was strongly influenced by exposure to wastewater. A significant enhancement relative to the positive control ($P < 0.05$) was evident after two weeks. Subsequently, the effect of exposure to wastewater was negative, i.e., removal efficiencies for the exposed sands decreased with time (Fig. 4-6). Such negative effect was intensified by biogrowth accumulation (as indicated by protein content and SEM) on

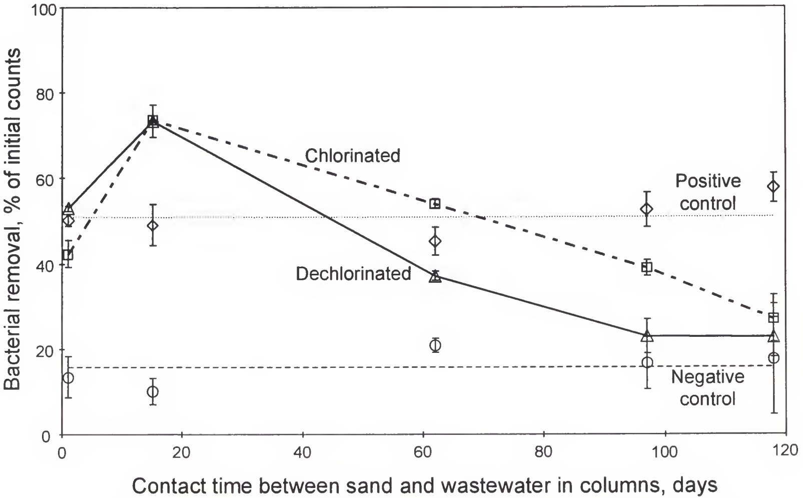

Fig. 4-6. Batch removal of *E. coli* by aluminum hydroxide-coated sand exposed to dechlorinated or chlorinated wastewater. Positive control = aluminum hydroxide-coated sand that was not exposed to wastewater; negative control = uncoated sand that was not exposed to wastewater. Error bars represent ±1.0 S.D.

sand exposed to dechlorinated wastewater. Enhancement of bacterial removal ("conditioning effect") upon short-term exposure of granular media to natural waters was observed by Schneider et al. (1994). Our preliminary work (Fig. 4-7) gives trends in bacterial removal and biogrowth (as measured by protein) that are consistent with results of our long-term experiments.

Overall trends seen in the batch tests were in good agreement with the column results shown in Figure 4-8. After 1 day of exposure to wastewater, all the coated sands removed approximately the same amount of bacteria (50-55%), significantly exceeding the removal by the uncoated sand (14%). By day 118, removal by the coated sands exposed to wastewater had decreased to a level near that achieved by the uncoated sand (negative control). In fact, the exposed sands performed equivalently to the uncoated sand at this time, whereas the coated sand that was not exposed to wastewater maintained a removal of approximately 55%.

After the initial conditioning period, sand with biogrowth had a significantly lower bacterial removal capacity than sand without biogrowth ($P < 0.05$) (Fig. 4-6). Removal efficiency for sand with biogrowth declined to the level of the negative control sooner than sand without biogrowth (97 vs. 118 days). Others have reported improved removal of bacteria (Banks & Bryers, 1992) and bacterium-sized particles (Drury et al., 1993; Rittmann & Wirtel, 1991; Sprouse & Rittmann, 1990) by biofilm-coated media. The contradictory results of our study may be attributable to differences in the substratum (sand in our study vs. glass, polycarbonate, or granular activated carbon), particles removed (bacteria vs. latex beads or organic solids), and fluid medium (treated wastewater effluent vs. synthetic wastewater or artificial medium). The phenomenon of enhanced

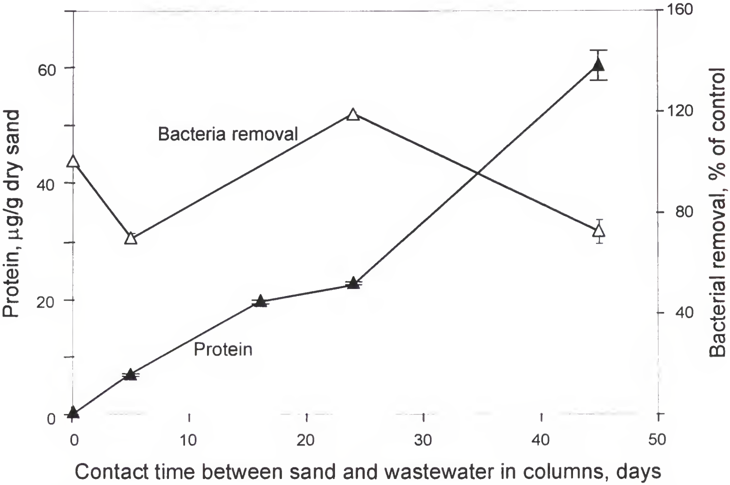

Fig. 4-7. Correlation between biogrowth (as measured by protein content) and batch removal of *E. coli* by aluminum hydroxide-coated sand exposed to dechlorinated wastewater. The right axis gives the removal percentages of the test sand divided by the respective removal percentages of the positive control. Error bars represent ±1.0 S.D.

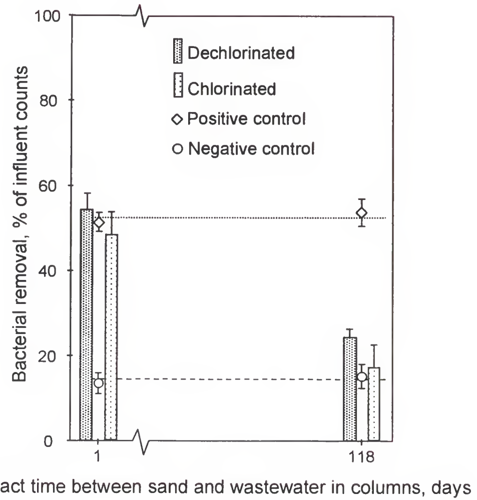

Fig. 4-8. Column removal of *E. coli* by aluminum hydroxide-coated sand exposed to dechlorinated or chlorinated wastewater. Positive control = aluminum hydroxide-coated sand that was not exposed to wastewater; negative control = uncoated sand that was not exposed to wastewater. Error bars represent ±1.0 S.D.

bacterial removal following initial exposure of sand to wastewater may be effected by a "conditioning film" of adsorbed ions and other inorganic and organic colloids on the media surface (Schneider & Marshall, 1994).

Changes in zeta potential do not appear to explain the decreased removal capacity of the sand in the presence of biogrowth, as opposite trends were observed in zeta potential (Fig. 4-5) and bacterial removal (Fig. 4-6) of sand with biogrowth. Normally, increased positive character of zeta potential would be expected to enhance bacterial removal (van Loosdrecht et al., 1990). Reduced removal capacity also does not seem to be consistent with the expected increase in hydrophobicity of sand surface due to biogrowth. For example, coating quartz surface with polyethyleneoxide (hydrophobic) enhanced removal of hydrophobic bacteria, but had a marginal (positive) effect on the removal of hydrophilic bacteria (McCaulou et al., 1994). The negative effect of biogrowth may be interpreted as blockage of attachment sites by the bacterial cells and associated exopolymers making up the biogrowth. For example, protein films on inanimate substrata have been found to generally inhibit bacterial attachment (Fletcher, 1976).

Since Al content and zeta potential did not change significantly after day 14, reduced removal capacity of Al-coated sand in the absence of biogrowth must be due to factors other than the loss of coating or decrease in surface charge. Loss of fine structures (on the order of 0.1 μm) of Al-coated sand surface was observed by SEM (6,000 ×) after 14 days of exposure to wastewater. This would suggest a decrease in surface area or surface roughness as a consequence of prolonged exposure to wastewater. Another possible mechanism for the loss of coating effectiveness is the saturation of attachment sites as a consequence of adsorption of macromolecules present in the wastewater.

Aging of porous media under continuous exposure to wastewater flow limits the effective lifetime of Al-coated sand ("effective" means possessing superior removal capacity vs. uncoated sand) to about 4 months. Biogrowth reduces the effective lifetime to approximately 3 months. This means that surface modification is suited for short-term applications. Long-term application is problematic, since it would require replacement or renewal of surface-modified porous media every 3 months to maintain effectiveness. For long-term application to be feasible, modification techniques must be developed to substantially improve the effective lifetime of surface coating. Reuse of porous media is also an alternative, but requires economical means of restoring coating effectiveness.

Conclusion

The aging phenomena of aluminum hydroxide-coated sand as porous media were examined in relation to bacterial removal. The coating progressively lost its enhancing effect when exposed to wastewater, although short-term (days to weeks) exposure has a reproducible "conditioning" (positive) effect on bacterial removal. Biogrowth adversely affected bacterial removal. Effective lifetime of Al-coated sand was 4 months without biogrowth and 3 months with biogrowth. Al content decreased by one quarter within the first two weeks, but no significant loss was observed thereafter. Neither decrease in Al content nor change in zeta potential could explain the loss of coating effectiveness. SEM suggests that loss of surface microstructure play a role in the aging effect of prolonged exposure to wastewater. These findings are of importance for assessing the economics as well as technological potential of surface modification as a means of improving the removal efficiency of granular media filters in water and wastewater treatment.

CHAPTER 5

EFFECT OF INFLUENT pH ON REMOVAL CAPACITY OF ALUMINUM HYDROXIDE COATED SAND

Introduction

Coating filter media with metallic hydroxides is an emerging strategy for increasing the removal of fine particles such as bacteria and viruses from water and wastewater (Ahammed and Chaudhuri, 1996; Gerba et al., 1988; Lukasik et al., 1996; Mills et al., 1994). Biocolloids (e.g., bacteria, viruses) carry a negative surface charge, as do most natural filter media (e.g., sand, diatomaceous earth), in the pH range of natural waters (Loder & Liss, 1985; Marshall, 1976; Raab & Stumm, 1993). Metallic hydroxide coatings serve to reduce the electrostatic repulsive forces by rendering the surface of the filter media more electropositive, thus facilitating colloidal attachment

Among concerns about this surface modification strategy is the sustainability of removal capacity when coated filter media are placed in service Influent characteristics such as pH can play an important role in the deterioration of the surface coating, since the dissolution behavior of $Al(OH)_3$ is pH-dependent (Snoeyink & Jenkins, 1980). Factors affecting the stability of the surface coating can also affect removal capacity. Since influent conditions may vary from one water or wastewater treatment plant to another, it is of both scientific and practical significance to understand the behavior of surface-modified filter media in response to specific fluid conditions. At present it is poorly understood how pH may affect removal capacity of metallic hydroxide-coated media.

The purpose of this study was to examine the effect of influent pH on performance of metallic hydroxide coating on filter media. Aluminum hydroxide-coated sand was used as the model system in parallel columns with continuous upflow of pH-adjusted tapwater. Performance of the coated sand was measured in terms of *Escherichia coli* removal. The results provide much needed information on the removal capacity and effective lifetime of the surface-modified sand in response to influent pH conditions.

Materials and Methods

Surface Modification of Sand

The fraction of 20 × 30 mesh Ottawa sand (Fisher Scientific, Springfield, NJ) passing through a U.S. Standard No. 25 sieve was collected to obtain sand particles in the size range of approximately 600-700 μm diameter. The graded sand was coated with aluminum hydroxide to enhance adsorption of bacteria (Lukasik et al., 1996) according to the following procedure. A 3,000-g batch of sand was rinsed with deionized water until the supernatant became clear, air-dried, then spread out to form a 5-cm thick layer in a glass tray. A volume of 1,000 mL of 1.0 molal $AlCl_3 \cdot 6H_2O$ (Fisher Scientific) solution was added to the tray. After 30 min of contact, the excess solution was drained and sand oven-dried in the glass tray at 65-70°C for 24 hr. The over-dried sand, while still warm, was transferred slowly into a 4.0-L glass beaker containing 2,000 mL of 3.0 N ammonium hydroxide and contacted for 10 min. After draining excess liquid, the sand was oven-dried again in the glass tray at 65-70°C for 24 hr, then stored at room temperature in a sealed plastic container. Immediately before column packing, the coated sand was rinsed with deionized water adjusted to pH 7.0 until the supernatant became clear to remove loose precipitates. A portion of this sand was saved for use as "positive control" in parallel with all column samples. Similarly, uncoated sand that was not exposed in the columns was used as the "negative control."

Exposure of Al-coated Sand to pH-adjusted Tapwater

Aluminum hydroxide-coated sand was exposed to pH-adjusted tapwater in acrylic columns (3.2 cm I.D. × 1.5 m) operated at room temperature (22-25°C) with continuous upward flow. The influent tanks were refilled daily with fresh tapwater adjusted to pH 5.0, 7.0 or 9.0, respectively, with HCl and NaOH. The flow rate in the columns was 57 mL min^{-1}, which gave a superficial velocity of 1.2 mm s^{-1} Columns and tubing were covered with black plastic to prevent growth of algae The columns were backwashed for 15 min weekly at a superficial velocity of 17 mm s^{-1}, which was sufficient to completely fluidize the sand. At scheduled intervals, the sand was emptied from each column, drained and thoroughly mixed, then samples were taken. Samples for batch removal tests were neutralized in pH-7.0 artificial groundwater (AGW) (McCaulou et al., 1994) and stored at 4°C for up to 24 h. The remaining sand was returned to the respective columns and feeding was resumed.

Preparation of Test Bacteria

Batch cultures of *Escherichia coli* C3000 (ATCC 15597) were grown overnight to early stationary phase in Nutrient Broth (Sigma Chemical, St. Louis, MO) at 35°C with shaking at 120 rev min^{-1}. A bacterial suspension of 50 mL from each batch was centrifuged at 3000 × g for 10 min at 4°C to harvest the cells. The cells were resuspended in filter-sterilized MilliQ water to $A_{550} = 0.40$ and stained for 30 min in complete darkness with 4', 6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980) at a final concentration of 25 μg/mL. Subsequently, the stained cells were harvested, washed in 2 × volume (100 mL) of AGW (pH 7.0) with vortexing, and re-harvested by centrifugation at 7000 × g for 10 min at 4°C. The freshly stained cells were resuspended in AGW (pH 7.0) and kept in complete darkness at 4°C until use.

Batch Removal Tests

Batch removal tests were carried out using samples of $Al(OH)_3$-coated sand from the test columns. Parallel tests were also run with $Al(OH)_3$-coated (positive control) and uncoated (negative control) sands that were not exposed to pH-adjusted tapwater. Initial bacterial concentrations were adjusted to 10^5-10^6 cells mL^{-1}. The test bacteria were contacted for 2 hr with the respective sands in 50-mL plastic centrifuge tubes affixed to a 70 cm diameter wheel that was rotated vertically at 30 rev min^{-1} at room temperature without light. Each test tube contained 5 g of wet sand (rinsed with AGW) and 20 mL of bacterial suspension. Blanks containing bacterial suspension but no sand were run along with the other tubes to obtain initial counts. After mixing, sand in the tubes was allowed to settle for 10 min at room temperature, then supernatants were sampled. Tests were run in triplicate. Samples were stored in complete darkness at 4°C until enumeration.

Bacterial Enumeration

Direct counts of bacterial samples were obtained within 48 hours (preliminary experiments showed that cell losses were less than 5% after this length of storage) using epifluorescence microscopy (APHA et al., 1992; Sherr et al., 1993). The samples were not re-stained, hence only pre-stained test cells were counted. This prevented interference from indigenous cells (background contamination or sloughed biomass). A Leitz Microlab microscope, equipped with appropriate filter blocks for desired light excitation and emission, was used for visualization and counting. Samples were vortexed vigorously to mix well immediately before filtration. A 1.0-mL aliquot of sample was filtered through a sterile black polycarbonate filter of 0.20 μm pore size (Costar Scientific, Cambridge, MA), with a 0.45 μm pore size (GN-6) membrane filter (Gelman Sciences, Ann Arbor, MI) placed underneath to evenly distribute the vacuum. Filters were pre-wetted with filter-sterilized MilliQ water and a vacuum of 600 mm Hg applied. Three mL of filter-sterilized MilliQ water was added to the filter funnel immediately before applying vacuum to help disperse bacteria on the filter. Ten microscopic fields were counted per filter. Sample concentrations typically gave 25-50 cells per microscopic field, hence the total number of cells counted per sample exceeded the minimum range recommended by Kepner & Pratt (1994).

Results and Discussion

Batch removal tests were carried out to assess the effect of influent pH on the capacity of coated sand to remove bacteria. Initial removal capacity of coated sand (positive control) was significantly better than uncoated sand (negative control) (approximately 50-70% vs. 10-20%). A significant decline of removal capacity was evident after 24 hr of exposure to tapwater at all pH levels (Fig. 5-1). The extent of this initial decline varied with the influent pH. The order of magnitude of the pH effects was, namely, pH 5.0 > pH 9.0 > pH 7.0. Subsequently, removal capacity steadily decreased to that of uncoated sand in 22 days for pH 5.0 and 9.0, and in 29 days for pH 7.0. The rates

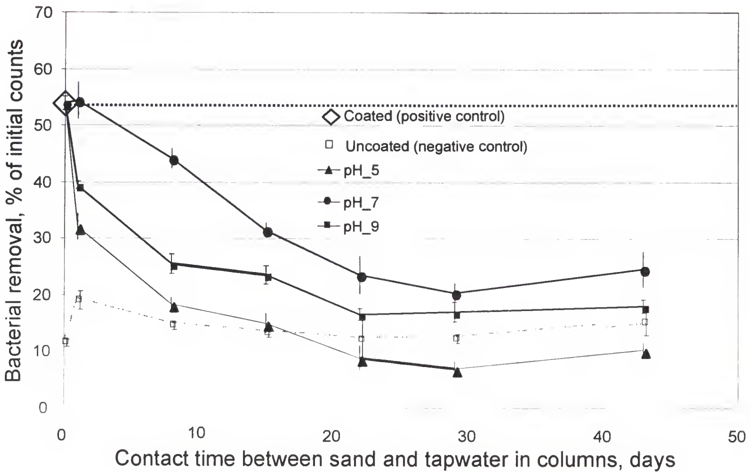

Fig. 5-1. Effect of influent pH on performance of aluminum hydroxide-coated sand exposed to tapwater - long-term experiment. Positive control = aluminum hydroxide-coated sand that was not exposed to tapwater; negative control = uncoated sand that was not exposed to tapwater. Error bars represent ±1.0 S.D.

of decline were similar for all pH levels, although at pH 5.0 the removal capacity dropped to below that of uncoated sand after 22 days.

The column experiment was repeated using a second batch of coated sand to assess the reproducibility of these pH effects. Samples were taken more frequently for the initial 24 hr (at 1, 6, 12 and 24 hours) to elucidate the initial patterns of the pH effects. As shown in Figure 5-2, pH-7.0 samples consistently outperformed pH-5.0 and pH-9.0 samples. The decrease in removal capacity was more pronounced at pH 9.0 than at pH 5.0 over the initial hour. Subsequently, the removals of pH-5.0 samples were statistically similar to those of pH-9.0 samples before the 24-hr point, thereafter removals of pH-5.0 samples became inferior to those of pH-9.0. These general trends were in agreement with those found in the first experiment, indicating reproducibility of the pH effects.

Effect of influent pH on removal capacity of coated sand may be explained by the pH-dependent dissolution of aluminum hydroxide precipitates on the sand surface. Since binding of Al^{3+} ions to mineral surfaces is by complex formation with the functional >OH groups on the surface, stability of the surface coating is determined more by the chemical interaction energy than by electrostatic forces (Raab and Stumm, 1993). Fluid pH can influence the chemical dissociation kinetics of the Al(OH) -surface complex and thus the stability of the surface coating. For example, release of Al^{3+} ions from $Al(OH)_x$-montmorillonite surfaces (chlorite-like complex on clay surface) was appreciable at pH 5.0 and 9.0, but was negligible at pH 7.0 (Violante & Gianfreda, 1993).

The trends in pH effects on removal capacity reported here coincide with the pH-dependent solubility of aluminum hydroxide. Removal capacity of Al-coated sand was affected in order of magnitude by pH 5.0 > pH 9.0 > pH 7.0. Similarly, the solubility of

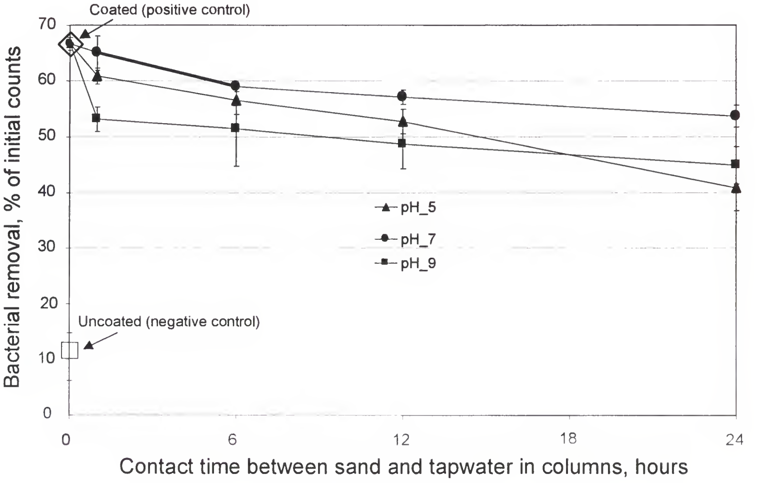

Fig. 5-2. Effect of influent pH on performance of aluminum hydroxide-coated sand exposed to tapwater - short-term experiment. Positive control = aluminum hydroxide-coated sand that was not exposed to tapwater; negative control = uncoated sand that was not exposed to tapwater. Error bars represent ±1.0 S.D.

$Al(OH)_3$ minimizes at pH 6.0-7.0 and maximizes at extreme pHs, more so in the acidic range than in the basic range (Snoeyink & Jenkins, 1980). In studying the dissolution behavior of aluminum hydroxide formed on α-alumina surface, Hirata et al. (1995) discovered that the amount of Al^{3+} dissolved in acidic suspensions (pH 2.6-5.5) was more than that in basic suspensions (pH 7.2-9.3), both containing Mg^{2+} ions. The interplay of pH and other solution factors is also possible in causing the decline of removal capacity. For example, Al^{3+} release from a $Al(OH)_x$-montmorillonite surface complex increased markedly with the addition of ligands (oxalate/phosphate) in the solution at pH 5.0 and 9.0, but not at pH 7.0 (Violante & Gianfreda, 1993).

Fluid pH can also influence the speciation of Al(OH) -surface complexes, which in turn could affect removal capacity. Raab and Stumm (1993) discovered that the speciation of Al^{3+}-silica surface complex depends upon solution pH: at $pH < 4.5$, free Al^{3+} predominates, while at $pH > 5.5$, hydroxo Al^{3+} complexes become predominant. Other possible mechanisms may include, but not limited to, decrease in surface area and roughness, change in surface microstructure, and loss of attachment sites due to chemical fouling, all of which may or may not be related to fluid pH.

In water and wastewater treatment, fluid pH can vary greatly from one plant to another, depending on the influent source, system design, and operating conditions. Even within a plant, water pH can fluctuate considerably, due to diurnal or seasonal variations. The pH range (5.0 to 9.0) tested in this study should cover pH conditions encountered at most water and wastewater treatment plants. Understanding the effect of pH is important from an economic as well as technological perspective, since pH can influence both removal capacity and life expectancy of surface-modified filter media such as sand.

Conclusion

This study has shown that the removal capacity of aluminum hydroxide-coated sand can be influenced strongly by influent conditions such as pH Among the 3 pH levels tested (pH 5.0, 7.0 and 9.0), neutral pH had the least effect on removal capacity, although the rates of decline of removal capacity after an initial 24-hr period were similar for all 3 pH levels. The pH effects were, in order of magnitude, pH 5.0 > pH 9.0 > pH 7.0. These trends were reproducible. The effective lifetime (in terms of *E. coli* removal) of aluminum hydroxide coating on sand was less than one month at the acidic and basic pHs, and about one month at the neutral pH.

CHAPTER 6
CONCLUSIONS

The aging of surface-modified filter media is of both economical and technological concern. Albeit a complete understanding of aging phenomena would require further investigation, the experimental results nevertheless warrant some tentative conclusions:

- Short-term (days to weeks) exposure of aluminum hydroxide-coated sand to wastewater has a reproducible "conditioning effect" (positive effect) on bacterial removal, while prolonged exposure leads to progressive decline of coating effectiveness.
- Removal capacity of surface-modified sand exposed to wastewater is negatively affected by biogrowth, but does not appear to be closely correlated with the loss of coating or change in zeta potential.
- The effective lifetime of surface-modified sand as filter media is approximately 4 months without biogrowth and 3 months with biogrowth under the experimental conditions employed.
- Water pH is an important factor in the aging process of surface-modified sand. The effect of influent pH on removal capacity appears to be consistent with the pH-dependent dissolution behavior of aluminum hydroxide.
- The effective lifetime of aluminum hydroxide-coated sand exposed to tapwater is in the range of 1 month (in order of longevity, pH 7.0 > pH 9.0 > pH 5.0).

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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